

14 December 2012 | \$10

Science



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COVER

Arachnoscelis magnifica, a predaceous katydid (Orthoptera: Tettigoniidae), on a leaf in the San Lorenzo forest of Panama. This attractive insect is but one of the ~25,000 arthropod species likely to live in this 6000-hectare forest, confirming the formidable diversity of arthropods, which appears to be 300 times that of mammals in tropical rainforests. See page 1481.

Photo: Maurice Laponce, Royal Belgian Institute of Natural Sciences

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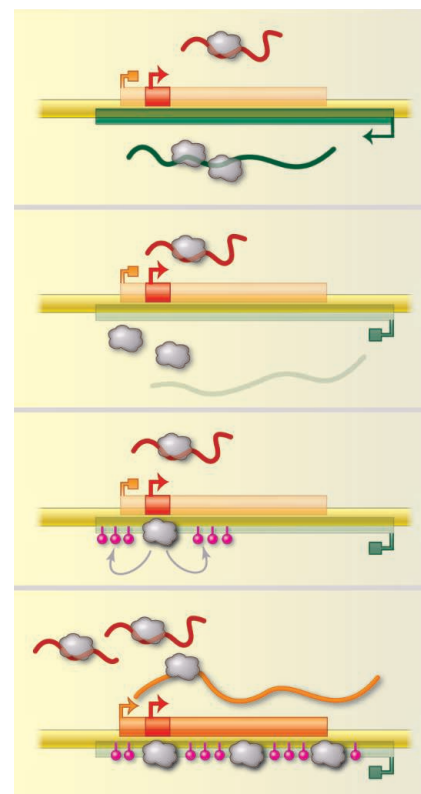
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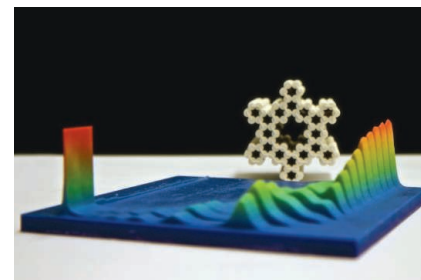
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J. Oliver-Meseguer et al.
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- 1458** Rapid Folding of DNA into Nanoscale Shapes at Constant Temperature
J.-P. J. Sobczak et al.
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P. A. Latos et al.
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The mechanism by which removal of signals from the reproductive organs promotes longevity in nematodes is elucidated.
- 1476** *Hox* Genes Regulate Digit Patterning by Controlling the Wavelength of a Turing-Type Mechanism
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ONLINE HIGHLIGHTS

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Publication Ahead of Print

Variable Clonal Repopulation Dynamics Influence Chemotherapy Response in Colorectal Cancer

A. Kreso et al.

10.1126/science.1227670

Actin, Spectrin, and Associated Proteins Form a Periodic Cytoskeletal Structure in Axons

K. Xu et al.

10.1126/science.1232251

EF-P Is Essential for Rapid Synthesis of Proteins Containing Consecutive Proline Residues

L. K. Doerfel et al.

10.1126/science.1229017

Translation Elongation Factor EF-P Alleviates Ribosome Stalling at Polyproline Stretches

S. Ude et al.

10.1126/science.1228985

A Stringent Limit on a Drifting Proton-to-Electron Mass Ratio from Alcohol in the Early Universe

J. Bagdonaitė et al.

10.1126/science.1224898

SCIENCENOW

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Highlights From Our Daily News Coverage

Diuretic Drug Offers Latest Hope for Autism Treatment

In a small trial, bumetanide improves social interactions in some children with this disorder.

<http://scim.ag/Autism-Treatment>

Tree Rings for Lobsters

Newly discovered growth bands reveal crustacean ages for the first time.

http://scim.ag/Crustacean_Ages

Turning Pull Into Push?

One physicist puts a curious twist on the interactions of charge and surface.

http://scim.ag/Charge_Surface

SCIENCE SIGNALING

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The Signal Transduction Knowledge Environment

11 December issue: <http://scim.ag/ss121112>

RESEARCH ARTICLE: TIM Family Proteins Promote the Lysosomal Degradation of the Nuclear Receptor NUR77

S. Balasubramanian et al.

The TIM family of receptors attenuates cell death in kidney cells after injury.

RESEARCH ARTICLE: Mechanism of Stretch-Induced Activation of the Mechanotransducer Zyxin in Vascular Cells

S. S. Babu et al.

Multiple steps drive the stretch-induced nuclear translocation of the mechanotransducer zyxin.

MEETING REPORT: Celebrating 30 Years of Wnt Signaling

F. Verkaar et al.

Researchers gathered to share the latest findings on Wnt signaling in development and disease.

LETTER: Comment on "Epidermal Growth Factor Receptor Is Essential for Toll-Like Receptor 3 Signaling"

B. Burtress et al.

Targeted inhibitors used in cancer treatments may have adverse effects on immune responses.

SCIENCE TRANSLATIONAL MEDICINE

www.sciencetranslationalmedicine.org

Integrating Medicine and Science

12 December issue: <http://scim.ag/stm121212>

FOCUS: T_{reg} Cells in Cancer—A Case of Multiple Personality Disorder

P. D. Bos and A. Y. Rudensky

RESEARCH ARTICLE: Expression of ROR γ t Marks a Pathogenic Regulatory T Cell Subset in Human Colon Cancer

N. R. Blatner et al.

T_{regs} that expand in human colon cancer have proinflammatory properties and contribute to tumor progression.

PERSPECTIVE: Pluripotent Stem Cells—Immune to the Immune System?

J. I. Pearl et al.

The immunogenicity of pluripotent stem cells will dictate the type of immunosuppression needed for cell therapy.

RESEARCH ARTICLE: Dantrolene Enhances Antisense-Mediated Exon Skipping in Human and Mouse Models of Duchenne Muscular Dystrophy

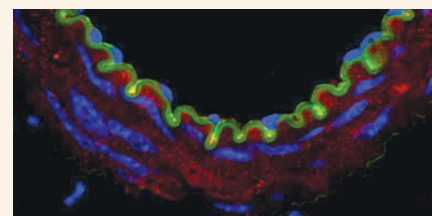
G. C. Kendall et al.

A ryanodine receptor antagonist facilitates exon skipping for treatment of Duchenne muscular dystrophy.

RESEARCH ARTICLE: Ser¹²⁹² Autophosphorylation Is an Indicator of LRRK2 Kinase Activity and Contributes to the Cellular Effects of PD Mutations

Z. Sheng et al.

LRRK2 Ser¹²⁹² autophosphorylation may be useful for screening candidate LRRK2 inhibitors.



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SCIENCE CAREERS

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Big Hopes, Small Changes for Biomedical Workforce Training

M. Price

In implementing the recommendations of its workforce working group, NIH decides to play it safe.

Experimental Error: The Myth of the Well-Rounded Scientist

A. Ruben

Despite what grad school admissions committees seem to believe, outside interests are good.

Administrative Hurdles Delay Ph.D. Scholarships in Spain

E. Pain

Many Spanish students have been forced to start their doctorates without financial help.

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ADVANCING SCIENCE. SERVING SOCIETY

Long Noncoding RNAs

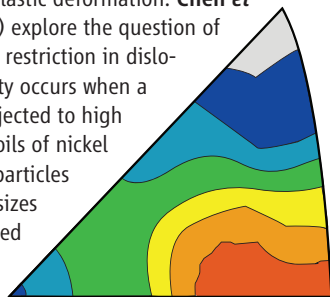
The past 5 years have uncovered thousands of long (>100 nucleotides) noncoding RNAs (lncRNAs) outpacing our understanding of their functions and mechanisms in regulating the genome. **Lee** (p. 1435) reviews the known and suspected means by which these intriguing molecules control gene expression locally and at great distances, considers potential universal roles for lncRNAs, and warns that classifying these intriguing molecules may be quite challenging given their diversity. The very long noncoding RNA, *Airn*, silences the *Igf2r* (insulin-like growth factor 2 receptor) imprinted gene cluster in mammals. **Latos et al.** (p. 1469) show in mouse cells that, rather than recruiting enzymes that modify histones to repress the locus, it is the act of transcription of *Airn* (and not the *Airn* gene product) that results in the silencing.

Spots to Remember

Scent marking is an essential component of communication for most mammals. Individuals remember the location of scent marks and regularly revisit marked sites, presumably to assess the condition and status of the animal doing the marking. It is known that individuals can follow odor or pheromone gradients to locate another individual, but relocating scent marks is a much more difficult task given the small amount of volatile compounds deposited, and their static nature. **Roberts et al.** (p. 1462) show that a nonvolatile component of male urine, the protein pheromone darcin, stimulates spatial preference and learning in mice. Female mice preferred locations where male urine (or synthesized darcin) had been found, and remembered these spatial locations for 2 weeks post-exposure.

Strength Under Pressure

Above a lower cutoff value, shrinking the grain size of a metal tends to strengthen it because the overall increase in grain boundaries limits the activity of the dislocations as the material undergoes plastic deformation. **Chen et al.** (p. 1448) explore the question of whether this restriction in dislocation activity occurs when a metal is subjected to high pressures. Foils of nickel made from particles of different sizes were subjected to high pressures



inside a diamond anvil cell. An increase in pressure extended dislocation activity to smaller grain sizes, indicating that pressure compensates for the inhibition of dislocation activity in small volumes.

From C=C to C=O

The palladium-catalyzed Heck reaction is widely used to form carbon-carbon bonds between aryl rings and olefins, after which elimination of a hydrogen atom restores the olefin's double bond. **Werner et al.** (p. 1455; see the Perspective by **Gilbertson**) present a variant of this reaction in which a hydrogen atom is instead lost from an alcohol center elsewhere in the molecule, yielding a ketone and a chiral center where the arene is bound. The process is highly enantioselective and also versatile: The alcohol can be sited one, two, or even three carbons away from the olefin.

So Different Yet So Similar

Gamma-ray bursts (GRBs) are associated with the collapse of stars into black holes. Blazars are a class of active galaxies powered by accretion onto central black holes with masses a million to a billion times that of the Sun. **Nemmen et al.** (p. 1445) show that, despite tremendous differences in luminosity and black hole mass, the relativistic jets produced in GRBs and blazars follow the same correlation between the kinetic power carried by the accelerated particles and the energy radiated away in the jet, suggesting that there may be a single mechanism for producing relativistic jets.

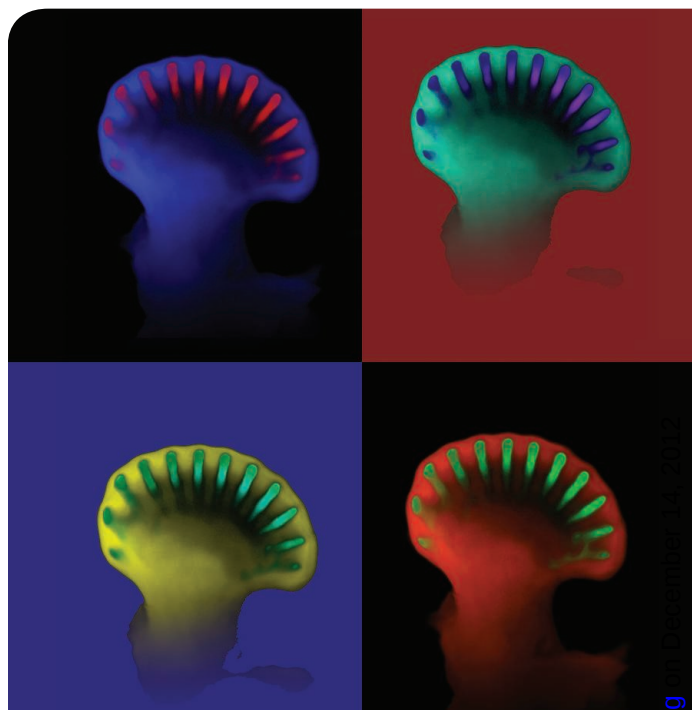
Speeding Up Nanoscale DNA Assembly

An impressive array of three-dimensional (3D) nanoscale objects has been assembled by folding a long, single-stranded DNA scaffold by binding of short DNA staples. However, these processes tend to be slow and inefficient. **Sobczak et al.** (p. 1458) examined the folding process with an intercalating fluorescence dye to

measure the formation of double-stranded DNA in folding processes or single-stranded DNA in unfolding. Reaction conditions were identified that sped up folding by orders of magnitude and increased yields of the 3D nanoscale objects to nearly 100%.

Assessing Creepy Crawlies

Arthropods are the most diverse group of terrestrial animal species, yet estimates of the total number of arthropod species have varied widely, especially for tropical forests. **Basset et al.** (p. 1481, see the cover) now provide more reliable estimates of total arthropod species richness in a tropical rainforest in Panama. Intensive sampling of a half hectare of forest yielded just over 6000 arthropod species. Scaling up this result to the whole forest suggests that the total species diversity lies between 17,000 and 40,000 species.



Digit Determination

Pentadactyly has been an early and rapid innovation of tetrapods. **Sheth et al.** (p. 1476) report that a dramatic reduction in distally expressed *Hox* genes, in the absence of a functional morphogen signaling pathway, results in extreme polydactyly in mice. Mutant digits exhibited patterns reminiscent of the endoskeleton of fins, suggesting that an ancestral patterning mechanism has been deeply conserved in evolution.

Resolving Redundancy

Many intracellular bacterial pathogens, like *Salmonella*, *Legionella*, and *Chlamydia*, make their homes within a host cell vacuole. Although we have cataloged many of the effector proteins secreted by these bacteria and their individual effects on host cell pathways, it is often not clear how the effectors work in concert to optimize bacterial growth. **O'Connor *et al.*** (p. 1440) tackled this complexity problem in *Legionella pneumophila*. This pathogen has an exceptionally long list of apparently redundant effector proteins to its name and, indeed, initial screening produced a library of 678 *Legionella* genes important for intracellular growth. Using bacterial mutagenesis to manipulate effector proteins and RNA interference in host cells to inhibit specific cellular membrane-trafficking pathways allowed systematic prediction by cluster analysis of sets of bacterial proteins with common targets required for intracellular growth.

Gold Cluster Catalysis

A variety of gold salts and complexes have been used to catalyze different organic reactions. Often, the catalytic rates for these reactions are similar. **Oliver-Meseguer *et al.*** (p. 1452; see the Perspective by **Hashmi**) observed an induction period for the onset of catalysis of organic reactions, such as the ester-assisted hydration of alkynes, for different gold salts and complexes. Mass spectrometry and absorption spectroscopy revealed that small gold clusters (three to ten atoms) formed during these induction periods and are likely to represent the active catalysts. The catalytic reaction rates could be extremely high—up to 10^5 turnovers of the catalyst per hour.

Alternative Role for EZH2

Epigenetic regulators are implicated in cancer progression and proposed as therapeutic targets. **Xu *et al.*** (p. 1465; see the Perspective by **Cavalli**) report that EZH2 (Enhancer of zeste homolog 2), a factor previously thought to exert its oncogenic function primarily as part of the polycomb repressive complex, acts through a distinct mechanism in cells of castration-resistant prostate cancer. Rather than exclusively silencing gene expression through histone methylation, EZH2 acts as a transcriptional coactivator. The activation function of EZH2 plays a critical role in the growth of castration-resistant prostate cancer cells, which could be relevant in future drug development.

Gonads and Life Span

Animals develop through successive life stages and have life spans that are determined by their genes and the environment. Yet how the molecular circuitry underlying life-stage structure relates to longevity is poorly understood. **Shen *et al.*** (p. 1472) now show that components of a steroid receptor-microRNA switch used for *Caenorhabditis elegans* developmental timing is co-opted to regulate adult longevity in response to signals from the reproductive system. Thus, the gonad can be coupled to metazoan life span via a developmental clock.



Bruce Alberts is Editor-in-Chief of *Science*.

Mobilizing Scientific Societies

IN LAST WEEK'S EDITORIAL, I LAMENTED THE SUPERFICIAL, SKIN-DEEP APPROACH TO SCIENCE LEARNING that is common in America's schools.* The situation has proven highly resistant to change, and it continues to have a disastrous, long-lasting effect on the attitudes of students toward science (millions of whom are now adults). The main culprit is the strong demand for a broad "coverage" of each subject, which kills student interest and makes genuine comprehension almost impossible. At the precollege level, this push is driven by state-based textbook adoption policies, by high-stakes examinations, and—inadvertently—by a scientific community that largely fails to understand teachers' needs. How might scientists be mobilized to support a much more inspiring, in-depth form of science education?

Many beautiful stories lie at the heart of science. Consider, for example, the beginning of life for an animal like ourselves. Somehow a single fertilized egg cell is able to multiply to give rise

first to a tiny embryo and then, through many cycles of cell growth and division, to an adult animal composed of thousands of billions of cells. This process requires that cells behave like tiny computers that store a memory of where they have been in the embryo, selectively expressing only those genes appropriate for their time and place in the giant "cell cooperative" that is a multicellular organism. Amazing time-lapse videos have been produced by researchers that could be enhanced with age-appropriate narrations to make this biology come alive. One need not know anything about the molecules or organelles inside cells, nor to be introduced to scientific terms like endoderm, mesoderm, and ectoderm, to appreciate the process of embryonic development that forms a human baby. Yet, a 700-page life science textbook for 12-year-olds selected by the state of California (with a glossary of 500 words) never challenges students to consider the fascinating question of what cells must do to produce an embryo, focusing instead on introducing them to

many hundreds of dry "scientific" terms and a multitude of associations to memorize.

If funds were devoted to quality research in schools to ascertain what students actually learn from curriculum materials, a textbook like this could never survive. Badly needed are materials for teachers that guide students to confront a phenomenon such as embryo development and then, working in small groups with skillful coaching, to imagine potential ways to explain it. After struggling with such a problem, students may progress far enough to appreciate Watson and Crick's elegant 1953 solution to the mystery of how cells can carry the huge amount of genetic information needed to produce a human being. But with no understanding of this mystery, a student's encounter with DNA becomes just one more item to be memorized in a deadly dull glossary of biology terms. This is but one example of why teachers need enough time to teach such fundamental concepts in depth, instead of being pushed to "cover" a subject such as biology in a single school year.*

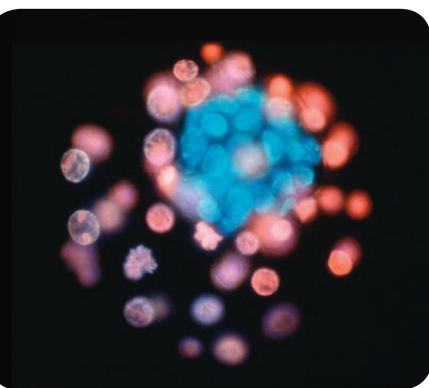
To facilitate such teaching, scientists will need to work in close partnership with outstanding teachers and other education experts—not only to research the effect of current curriculum materials and teaching methods on students (thereby advancing the science of education), but also to develop new, validated, Web-based curricula that address the critical national (and international) need for inspiring, in-depth lessons.

I propose that a set of scientific societies in different disciplines (covering biology, chemistry, physics, earth, and space sciences) be recruited for the above validation purposes. The publication of this issue coincides with the annual meeting of the American Society of Cell Biology in San Francisco, which is but one example of a large discipline-based organization with a strong interest in education,† ready, willing, and able to be called on to help change the current trajectory of science education in the United States and other parts of the world.

— Bruce Alberts

10.1126/science.1233700

*B. Alberts, *Science* **338**, 1263 (2012). †www.lifescied.org; www.ibioseminars.org.





ECOLOGY

Unlucky in Love

Some anurans, members of an order of amphibians that includes frogs and toads, are explosive breeders—males aggregate to attract females and compete aggressively for mating opportunities. This results in multiple males attempting to mount a single female, which may cause the female's death from the excessive weight. From an evolutionary perspective, this seems counterproductive, but analysis of the frog *Rhinella proboscidea* by Izzo *et al.* suggests that this may not always be the case. Males of this species were able to extract the eggs from females that expired as a result of these intense competitions among males. Field observations confirmed that males could manipulate dead females to extract oocytes and that these oocytes could be fertilized to produce embryos. These findings suggest that in some male-biased systems, due to the scarcity of available females and the intense competition for access to reproduction among males, this behavior may allow for fitness gains in both the male and female participants. In fact, males may actually experience positive selection for functional necrophilia. — LMZ

J. Nat. Hist. 10.1080/00222933.2012.724720 (2012).

CHEMISTRY

Stretching the Polanyi Rules

Forty years ago, Polanyi laid out a framework to explain and predict the relative effectiveness of translational and vibrational energy in driving the reactions of atoms with diatomics. The current frontier in state-resolved molecular dynamics research encompasses the reactions of halogen atoms with methane and its various isotopologues—a six-atom system. Over the past several years, successive experimental and theoretical studies have sought to clarify the extent to which the Polanyi Rules apply (or fail to apply) to such systems. For the reaction of chlorine with CHD₃, the Rules suggest that the product-like transition state ought to favor the efficacy of vibration over translation, although the results of recent studies have been ambiguous in this regard. Zhang *et al.* now report quantum dynamics simulations of this reaction and compare their results with those from previous experiments and quasiclassical trajectories. The simulations suggest that C–H stretch excitation does indeed promote the reaction, except at collision energies below 1 kcal/mol. — JSY

J. Phys. Chem. Lett. 10.1021/jz301649w (2012).

GEOLOGY

Recording the Doldrums

The winds in the “doldrums” region near the equator are rather calm, and marine settings there see barely any hurricanes. The relative stability of ocean currents and waves in this region minimizes disruption of seafloor sediments and benthic communities. This is likely to

have been true at other points in Earth history as well, such as the Late Ordovician, when there were also polar regions with permanent ice caps. To search for clues of a hurricane-free equator in the geologic record, Jin *et al.* examined a 6000-km cross section across the margins of what was then the continent of Laurentia—containing modern-day North America, Greenland, and



northern Europe. Massive carbonate deposits containing abundant *Thalassinoides* trace fossils and persistent brachiopod shell beds suggest environments in which shallow water with relatively few major hurricanes would host and/or preserve these fauna. The belt corresponds remarkably well to the equatorial region in paleomagnetism-based plate reconstruction models of the Late Ordovician, providing independent validation of such models. — NW

Geology 10.1130/G33688.1 (2012).

ASTRONOMY

Impulsive Activity

Comets and asteroids are normally distinguished on the basis of their composition, appearance, and orbits. Since 2006, however, nine bodies orbiting within the main asteroid belt have been found with physical characteristics similar

to those of comets; i.e., comae and narrow dust tails. They have been named main-belt comets and are still enigmatic because they are difficult to find and characterize. Moreno *et al.* used the Gran Telescopio Canarias, a 10.4-m telescope located in the Canary Islands in Spain, to observe the main-belt comet P/2012 F5 (Gibbs), which was discovered in March 2012—the ninth main-belt comet found. The optical data, obtained on 18 May and 8 June 2012, together with a model to characterize the dust environment around the comet, imply that an impulsive event lasting less than a day, possibly less than a few hours, occurred on 1 July 2011, with an un-

certainty of ± 10 days, producing the narrow dust trails observed in this comet. It is not possible to say what caused this event, but an outburst, a collision with another body, or a rotational disruption are some of the likely causes. Activity related to ice sublimation, which has been suggested for other main-belt comets, is unlikely, based on the properties of the dust. — MJC

Astrophys. J. 761, L12 (2012).

Continued on page 1398

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CELL BIOLOGY

Endocytosis In-filtration

A healthy human kidney filters about 180 liters of plasma per day, a highly regulated process that ensures the removal of toxins but the retention of proteins and red blood cells. Critical to this filtration process is the slit diaphragm, a cell-cell junction formed by specialized cells called podocytes that wrap around kidney capillaries. The slit diaphragm loses its structural and functional integrity in nephrotic syndrome, a disease characterized by protein loss in the urine (proteinuria) that can lead to kidney failure.

Studying genetically engineered mice, Soda *et al.* identified a role for clathrin-mediated endocytosis, a process implicated in membrane protein recycling, in the formation and maintenance of the slit diaphragm. Podocyte-specific deletion of any of several proteins essential for endocytosis caused severe proteinuria in the mutant mice. These results suggest that the podocyte cell-cell junctions may be more dynamic than previously thought. Endocytosis could be a mechanism that allows the slit diaphragm to be rapidly remodeled in response to physiological changes such as fluctuations in blood pressure and blood volume. — PAK

J. Clin. Invest. 10.1172/JCI65289 (2012).

ECONOMICS

Cap and Innovate?

Climate policies, such as emissions caps or carbon taxes, aim in part to induce technological change: Make emissions more expensive and market incentives will drive companies to develop technologies that emit fewer, or no, greenhouse gases. Such innovations, it has been argued, will drive down the costs of the policies. Gans suggests that prior research in this area gives an incomplete and sometimes misleading picture. His economic model explores three

types of technological innovations: fossil fuel efficiency (e.g., improved automobile efficiency), alternative energy (e.g., wind power), and carbon offset (e.g., carbon sequestration). As emissions caps drive down emissions-intensive behaviors, they make fossil fuel efficiency innovations less valuable, limiting incentives to improve technology. Alternative energy innovations may also suffer, as profit incentives would depend on the overall size of the economy, which could contract under emissions caps. A higher carbon price could, however, spur innovation in carbon offset technologies, suggesting that carbon offset credits should be widely integrated into emissions trading schemes. Gans concludes that the market alone, driven by climate policies, will not be enough. Innovation policies must be pursued as well, including public investments in fossil fuel efficiency and alternative energy. — BW

Am. Econ. J. Econ. Policy 4, 1 (2012).

PSYCHOLOGY

One If by Hand, Two If by See

Language is a human universal, yet differs across cultures. Reading and writing are learned skills, and it seems improbable that the rather different orthographies observed in French and Chinese would be processed in the same way in the brain. Nakamura *et al.* showed that readers of French do indeed differ on behavioral measures from readers of Chinese when confronted with words that are either static or handwritten with a forward or backward trajectory, and with either normal or squeezed spacing. The effect of spatial distortion was greatest for the Chinese participants when viewing the forward direction of handwriting motion, whereas the largest effect on the French participants occurred with static words. The neuroimaging results provided a similar double dissociation, in which repetition suppression (due to priming) was observed specifically in Exner's

area—a portion of the premotor cortex known to be activated during the viewing of motion—for the Chinese and in the visual word form area for the French. The authors suggest that these areas, along with those that were activated, but not differentially so, constitute a large-scale network that facilitates reading in all cultures, with relative cortical contributions dependent on whether one is reading by eye or by hand. — GJC

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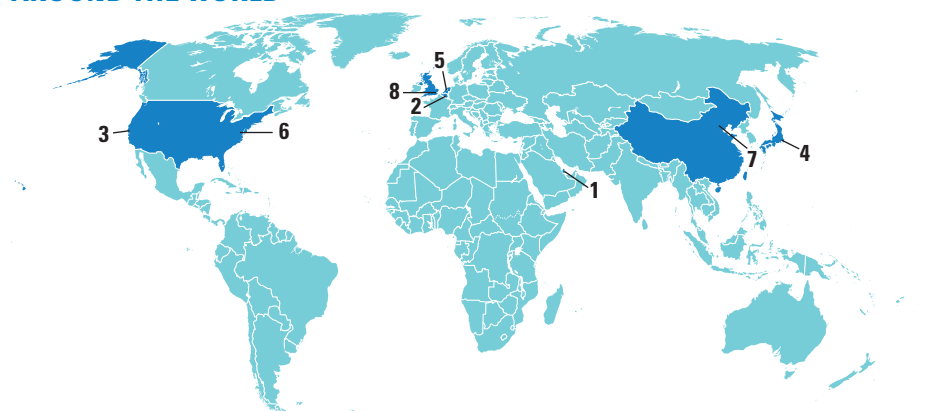
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Doha 1

Kyoto Protocol Hangs On

A 15-year-old global agreement to curb greenhouse gas emissions emerged weakened but still alive from the 18th United Nations conference on climate change, which ended on 8 December. The nearly 200 nations that attended agreed to extend the 1997 Kyoto Protocol by 7 years, to 2020. But several major emitters, including the United States, have never embraced the protocol, and others—including Japan, Russia, and Canada—dropped out in recent years. As a result, the renewed protocol covers industrialized nations responsible for just about 15% of global emissions—but delegates hope that



Deal. A U.N. official weighs climate options.

more nations will endorse a new agreement on emissions targets by 2015. Doha delegates also agreed to set up a new U.N. technology center to help developing nations curb emissions and prepare for climate change. However, they delayed for at least another year a plan for rich countries to begin to provide poorer nations with up to \$100 billion a year in climate aid by 2020. “It wasn’t pretty,” said Jennifer Morgan, director of climate programs at the World Resources Institute in Washington, D.C. “But Doha delivered just enough to keep the process moving.”

Brussels 2

E.U. Reaches Deal on Single Patent System

After decades of debate, the European Union seems poised to get a unified patent system that will make it easier and cheaper for researchers, institutions, and companies to protect inventions across borders. On 10 December, ministers of 25 member countries endorsed a new package to create the unitary patent; the next day, the European Parliament approved the complex deal as well. Michel Barnier, the European Union’s internal market commissioner, appeared confident on Monday that remaining hurdles—including an agreement to set up a new patent court—will be cleared in 2013.

Currently, the European Patent Office in Munich, Germany, handles bundle patent filings in up to 38 countries through a series of national procedures. The new regime will provide a one-stop shop and will lower the cost of an E.U. patent from about €36,000 to about €6400, according to the European Commission. As a result of complex negotiations, the patent court’s main seat will be located in Paris; secondary seats will be in London, for drug patents in particular, and Munich, for mechanical engineering filings. <http://scim.ag/EUonepatent>

San Francisco, California 3

CIRM Urged to Reorganize

The California Institute for Regenerative Medicine (CIRM) has provided a creative new source of funding that has turned the state into an international hub of stem cell research, but the agency could do a lot more to rid itself of possible conflicts of interest and other organizational problems. That was the take-home message last week from an

Institute of Medicine (IOM) panel that CIRM had commissioned to review its operations.

The stem cell agency was created by a 2004 ballot initiative that raised \$3 billion for research through bond sales. One sore point among CIRM critics is that many members of its 29-member governing board represent institutions that have received CIRM grants. The IOM panel recommended that board members be excluded from grant review committees, and it suggested curtailing the board’s role in CIRM’s daily operations. It also recommended diversifying the board with more independent voices and more industry representatives, whose help will be needed to achieve CIRM’s stated goal of developing stem cell treatments. The panel also urged CIRM to formulate a plan to ensure financial sustainability after spending its initial funding. http://scim.ag/CIRM_reorganize



Mukojima, Japan 4

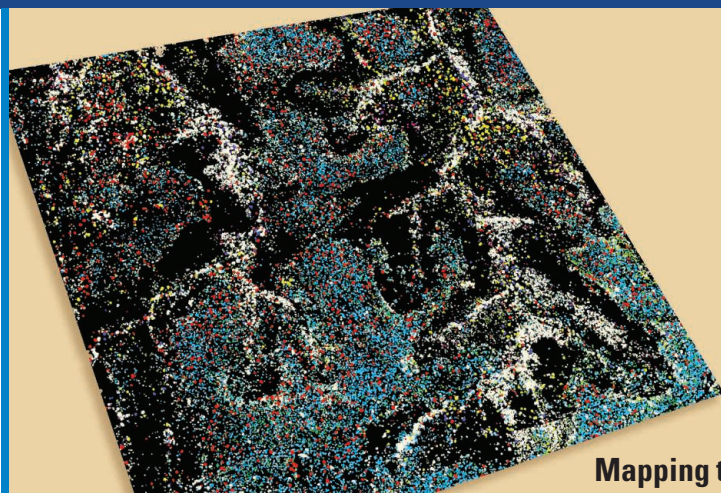
Albatrosses Colonize New Breeding Site

After years of waiting, Japanese efforts to reintroduce short-tailed albatrosses to a historic and safer breeding site paid off. Last week, researchers reported that they had found the first egg known to result from the mating of an adult that had been translocated as a chick to this new site. Since 2008, researchers have been taking chicks from Torishima, where an established breeding colony is considered threatened by an active volcano, and hand-raising them on Mukojima, 350 kilometers to the south. Short-tailed albatrosses, considered vulnerable by the International Union for Conservation of Nature, spend several years at sea before returning to their natal island to mate.

“It’s a very important development” in reintroducing the birds to Mukojima, says conservation biologist Kiyooki Ozaki at the Yamashina Institute for Ornithology in

Species:

- *C. apiculatum*
- *S. birrea*
- *Acacia*
- *C. collinum*
- *P. violacea*
- *E. divinorum*
- *T. sericea*
- *C. hereroense*
- Unknown



Mapping the Biosphere

The first images released from the Carnegie Airborne Observatory (CAO) wowed scientists last week at the American Geophysical Union fall meeting in San Francisco. Combining LiDAR and 428 bands of spectral data, the \$11 million system has scanned millions of hectares of forest since its deployment in 2011, most over the jungles of Central and South America. The system allows identification of individual tree species by their chemical signatures. (Each species is color coded in this image.) It can also determine tree size and health. CAO data-based presentations at the meeting explored a variety of topics, including the effects of drought on forests and the links between geology and jungle biodiversity. "It's a quantum leap from crude to sophisticated," says forest ecologist Glenn Juday of the University of Alaska, Fairbanks. Juday uses ground surveys to painstakingly characterize forest plots a meter at a time. "I'm afraid it will put me out of business. ... At other times, I'm excited about the opportunities." Gregory Asner, a global ecologist with the Carnegie Institution for Science in Palo Alto, California, who conceived CAO, says: "Welcome to the future." <http://scim.ag/biospheremap>

Abiko. The chick should hatch in January and fledge in May. Ozaki says they hope their techniques and experience can be applied to other sites and species.

Tilburg, the Netherlands 5

Social Psychologists Protest Stapel Report 'Attack'

The final report on the investigation into Diederik Stapel's fraud has itself come under fire from fellow social psychologists, who claim its conclusions about their field as a whole are too sweeping. An 8 December

statement from the Executive Committee of the European Association of Social Psychology (EASP) calls some of the report's conclusions "defamatory, unfounded, and false." And social psychologist Wolfgang Stroebe of Utrecht University in the Netherlands demanded an apology from the three investigation panels in a piece he wrote for his university's magazine.

The Stapel report, which has won praise for its rigor and transparency (*Science*, 7 December, p. 1270), says it's "unable to make any statement" about social psychology as a whole, but makes an "attack" on the field anyway, the EASP statement charges. For instance, the report says, "there are certain aspects of the discipline itself that should be deemed undesirable or even incorrect from the perspective of academic standards and scientific integrity." The objection is "unjustified," says the report's chief author, Willem Levelt of the Max Planck Institute for Psycholinguistics in Nijmegen, adding that he hopes social psychologists will "engage in some degree of self-reflection." <http://scim.ag/Stapelbacklash>



Stapel

NOTED

>After its scientists had worked for 2 years at a nearby university's labs, the Max Planck Florida Institute for Neuroscience officially opened its new, Jupiter, Florida—facility last week. It is the first U.S. outpost of the Germany-based Max Planck Society, which is a major supporter of basic research. At the opening ceremony, a German research minister pledged \$10 million annually over the next 4 years to the new institute.

THEY SAID IT

"Saying that I made any dent in the exploration of the trenches of the world would be like dropping out of an airplane into a cornfield at night and doing a 2-kilometer walk and saying I explored America."

—James Cameron, explaining at a press conference at the American Geophysical Union fall meeting that we've barely seen anything of the hadal depths of the ocean.

Washington, D.C. 6

NASA Lost in Space

A new report released last week by the National Research Council says that NASA doesn't know where it's going. The lack of "a national consensus on strategic goals and objectives" has set NASA adrift and is preventing the agency from forging a clear path ahead, the report says.

"A current stated interim goal of NASA's human spaceflight program is to visit an asteroid by 2025," said Albert Carnesale, a mechanical and aerospace engineer at the University of California, Los Angeles, who chaired the committee that authored the report. "However, we've seen limited evidence that this has been widely accepted as a compelling destination by NASA's own work force, by the nation as a whole, or by the international community."

The report recommends that Congress, the White House, and NASA pursue a number of options. Those options include restructuring the space agency's programs to reduce infrastructure and personnel costs; finding ways to partner with other agencies, the private sector, and international partners; increasing NASA's budget; and shedding programs that don't fit NASA's current budget profile. <http://scim.ag/NASAGoals>

Beijing 7

Chinese Investigation Slams 'Golden Rice' Study

The Chinese Center for Disease Control and Prevention (China CDC) last week released the results of an investigation into a controversial, U.S.-funded experiment involving feeding genetically modified (GM) rice to Chinese schoolchildren. >>>



Thirsty Cacti Collect Fog on Spines

As any desert hiker knows, cactus spines are one of nature's most effective deterrents. But this defense system can also act as outdoor plumbing. To survive drought in the southwestern United States, the cactus *Opuntia microdasys* uses clusters of spines like the one pictured to efficiently trap fog. Lei Jiang of the Institute of Chemistry of the Chinese Academy of Sciences in Beijing and his colleagues reported last week in *Nature Communications*. Using a scanning electron microscope, the researchers spied barbs on the tip of the spine and grooves along the middle that get wider as they get closer to the base of the spine. The barbs snag individual fog droplets, which coalesce into slightly larger droplets that move toward the base of the spine, they reported. Near the base, drops from adjacent spines meet up at outgrowths called trichomes, which funnel and absorb the water. Studying these details "offers new ideas [for] designing and fabricating artificial continuous water collectors," Jiang says.

with cancer and rare diseases.

Researchers have welcomed the new funding. "The announcement today of an additional £600 million of capital investment will hopefully help ensure that our world leading scientists have world leading facilities with which to work," said Royal Society President Paul Nurse in a statement.

But some say the money will simply make up for earlier cuts. The governing coalition cut capital spending in 2010 and froze the science budget at \$7.4 billion, so its value has since been eroded by inflation. The government has made up some of that shortfall with chunks of new funding over the past 2 years, including this new pot of money. "We were hoping that [Osborne] would continue his trend of supporting science and engineering. ... [T]he total amount of new funding since 2010 has now reached almost £2 billion [\$3.2 billion]," said Imran Khan, director of the Campaign for Science and Engineering, an advocacy group. <http://scim.ag/UKres>, <http://scim.ag/UKseq>

NEWSMAKERS

Former NASA Official Promises Private Moon Flights

NASA's former science chief, **Alan Stern**, says he can fly you to the moon by the end of the decade. Anticipated price: \$750 million. Stern, a planetary scientist who 4 years ago briefly served as the associate administrator for science at NASA, has launched a start-up, Golden Spike Co., that aims to offer commercial flights to the moon (and back) starting in 2020. "We realize this is the stuff of science fiction," Stern said at a news conference in Washington, D.C., last week. "We intend to make it science fact."



Stern

Stern made headlines in 2008 by making a sudden exit from his job at NASA after disagreements with then-NASA Administrator Michael Griffin over managing the space agency's science budget. His money management will be tested as CEO of the new venture, which will require billions of dollars in start-up costs. Stern says the company expects to get bookings from not just billionaire clients looking for an adventure but also from researchers and astronauts from countries interested in lunar science.

>>AROUND THE WORLD

A statement by the China CDC alleges that the investigators who conducted the study did not furnish parents with complete informed consent forms, concealed the fact that the children would be fed GM rice, and violated the Chinese health ministry's ethics policy on biomedical research. The three China-based investigators on the study have been removed from their posts.

The China CDC statement alleges that corresponding author Tang Guangwen of Tufts University in Boston violated Chinese regulations and brought cooked rice into China without obtaining proper approvals. Tufts University is now conducting its own investigation into the study; officials there declined to comment until that investigation is finished.

The study, funded by the U.S. National Institute of Diabetes and Digestive and Kidney Diseases and the U.S. Department of

Agriculture, was designed to test vitamin A absorption from "golden rice," an engineered variety high in β -carotene. Publication of the study by *The American Journal of Clinical Nutrition* in August sparked an uproar in China after Greenpeace China disseminated a press release calling it "a scandal of international proportions" (*Science*, 14 September, p. 1281). <http://scim.ag/goldrice>

London 8

New Money for U.K. Facilities

The United Kingdom's finance minister, George Osborne, last week announced £600 million (\$965 million) of new money for research over the next 3 years. The funding will be spent on big data and energy efficient computing, synthetic biology, and advanced materials. It also includes £100 million (\$160 million) to sequence the genomes of 100,000 Britons

BY THE NUMBERS

880 Latest census of mountain gorillas, an increase of at least 100 since 2006.

\$3,000,000 Fundamental Physics Prize money awarded to Stephen Hawking and to the CERN team who helped find the Higgs-like particle, respectively.

FINDINGS

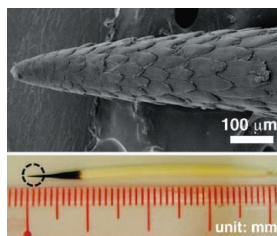
Using Satellites to Detect Buried Nuclear Blasts

Global satellite networks could become a powerful new tool for detecting clandestine underground nuclear explosions. Those explosions send up giant electromagnetic pulses that ripple through Earth's ionosphere, the portion of the upper atmosphere that is ionized by solar radiation. Although in theory, global satellite networks and radio telescopes should be able to spot the pulses, global monitoring efforts have instead relied on seismic, hydroacoustic, and radionuclide detection networks because earthquakes and major storms also cause ionospheric ripples. Last year, geodesist Dorota Grejner-Brzezinska and postdoc Jihye Park at Ohio State University, Columbus, and their colleagues pinpointed the 2009 nuclear blast in North Korea from these ripples. Last week at the fall meeting of the American Geophysical Union in San Francisco, they described algorithms for both GPS satellites and radio telescopes that enabled them to home in on two underground tests in the United States in 1992. However, even after the researchers work out the kinks, it's not clear that countries would add this technology to their monitoring efforts given that the Comprehensive Nuclear-Test-Ban Treaty has yet to be fully ratified. <http://scim.ag/satnucl>

Porcupine Quills Could Inspire Better Medical Devices

The secret behind a porcupine's painful quills are tiny barbs that help quills go in smoothly and not come out. Research into the function of these barbs could lead to improvements in bandages and hypodermic needles, says Jeffrey Karp, a bioengineer at Harvard Medical School in Boston. His team plunged barbed quills from North

American porcupines, quills whose barbs they had sanded off, quills from an African porcupine that naturally lack barbs, and a quill-sized hypodermic needle into samples of pig skin, measuring how much force it took to pierce the flesh and then how much force was required to extricate the quill. To the researchers' surprise, barbed quills required only about half as much force as the barbless quills (whether naturally barbless or sanded



Prickly. Tiny barbs coat the tips of porcupine quills.

clean) or the hypodermic needle did to breach the skin, they reported this week in the *Proceedings of the National Academy of Sciences*. Possible

applications: wound dressings attached with tiny barbed needles instead of potentially allergenic chemical adhesives, and hollow versions of quill-inspired needles in drug-delivery patches. <http://scim.ag/porquills>

Random Sample

Obamadon's Successors Got Postextinction Bounce

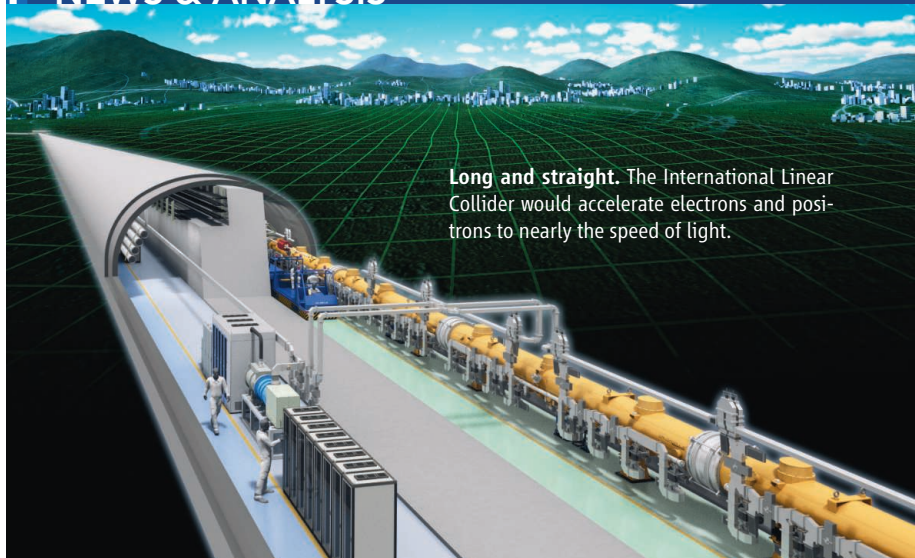
President Barack Obama survived a tough reelection battle this year—but scientists say an ancient lizard named for him met a far crueler fate. *Obamadon gracilis*, discovered in ancient rocks in Montana about a year ago, is one of dozens of lizards and related species newly described in a study published online on 10 December in the *Proceedings of the National Academy of Sciences*.

The 10-kilometer-wide asteroid that struck Earth 65 million years ago carved out the Gulf of Mexico and killed off the dinosaurs (except birds). Scientists thought lizards, snakes, and other so-called squamates mostly survived the Chicxulub impact—but new results from a team led by Nicholas Longrich of Yale University show that more than 80% of squamates also died in the mass extinction, including *O. gracilis* (blue lizard in foreground of this artist's rendition).

Mammals' evolutionary explosion, which began within 1.5 million years after the impact, is well documented—but it turns out the squamates had their own postimpact evolutionary bounce, Longrich says. By comparing hundreds of species of lizards and their kin before and after the impact, the team uncovered both the squamates' mass extinction and the rise of new species of squamates about 10 million to 15 million years later. Earlier researchers may have overlooked these evolutionary changes because the new squamates filled the same niches as the old ones, Longrich says: "It's like a play where they've changed all the actors, but the play's the same."

The finding gives a rare look at the before-and-after of an extinction event, says Stephen Brusatte of the American Museum of Natural History in New York City and Columbia University. *Obamadon* or not, he says, "a paper like this doesn't need a celebrity name" to grab the spotlight.





Long and straight. The International Linear Collider would accelerate electrons and positrons to nearly the speed of light.

HIGH-ENERGY PHYSICS

Japan Stands Alone in Bid For \$10 Billion Collider

TOKYO—Almost 2 decades before the Large Hadron Collider (LHC) at CERN caught a glimpse of the long-sought Higgs boson (*Science*, 13 July, p. 141), physicists began planning a machine that would examine the Higgs in detail and probe for even more elusive particles. On 15 December, scientists will celebrate the completion of the technical design report for their dream machine, the International Linear Collider (ILC). The next big hurdle will be figuring out where to build it and who will pay for it.

The contest seems to be Japan's to lose, as no other nation has shown much interest. In October, the Japan Association of High Energy Physicists floated a proposal to host the ILC, build it in stages, and foot half the total cost, which the Japanese estimate to be about \$10 billion. (Other estimates that have included contingency funding, inflation, and other costs have varied widely, and U.S. Energy Secretary Steven Chu once used a figure of \$25 billion.)

"If other countries contribute 50%, I think [the Japanese government] will support it," says Satoru Yamashita, a University of Tokyo physicist involved in the design. But it remains to be seen if the rest of the world will fall in line in time to start construction in 2015 or 2016, as physicists hope.

Unlike the LHC, which accelerates protons around an underground, 27-km, circular racetrack, the ILC will send particles in a straight line through a 31-kilometer-long tunnel. Its superconducting magnets

will accelerate electrons and their anti-matter counterparts, positrons, to a hair shy of the speed of light and smash them together at energies of 500 billion electron volts (GeV). Particles traveling around a curve lose energy to synchrotron radiation, and the lighter the particle, the greater the energy loss.

Researchers in Europe, Japan, and the United States started working on linear colliders separately but joined forces in 2004. Including those working on ILC detectors, the 1500 researchers at 300 laboratories and universities in 35 countries "are literally scattered across the globe with no lead lab," says Michael Harrison, a physicist at Brookhaven National Laboratory in Upton, New York, who is regional director for the Americas on the design team. "It is the first time that a high-energy physics facility has been designed by an international collaboration," he says.

Outside experts will review the technical design and come up with an official price tag sometime next spring. The technical design will also be the basis for valuing the proposed contributions of different parties.

To lower the initial outlay, the Japanese strategy is to "start small and grow," Yamashita says. The collider would start off as a "Higgs factory" with an energy of 250 GeV. Producing an abundance of Higgs bosons, which convey mass to fundamental particles, would allow researchers to study "the properties of the particle at a level of precision that goes well beyond the reach of the Large Hadron Collider," says Eckhard

Elsen, a physicist and member of the ILC project team at DESY, Germany's high-energy physics lab in Hamburg. Physicists hope that these studies will determine the mass and spin of the Higgs and how it interacts with other particles.

A staged upgrade to the 500 GeV energy target would enable studies of the top quark, dark matter particles, and possibly even extra dimensions of space. The machine could be extended to 1 trillion electron volts. Going to higher energies by extending the collider's length "is a big advantage of the linear design," Yamashita says.

In addition to doing physics, the project is expected to be an engine of Japanese regional development. The country has two candidate sites, one on Kyushu, the southernmost main island, the other in the Tohoku region in Iwate Prefecture, which was hit hard by the March 2011 tsunami. Another key factor will be the results of geological investigations now under way.

Japan's is the only proposal on the horizon. Although the U.S. Department of Energy has taken no official position on the ILC, Harrison says "the U.S. is not presently interested in hosting [a physics] facility of this size." And on 25 November, the German Committee for Particle Physics expressed "enthusiastic support" for the Japanese proposal. "[W]e strongly recommend [that Europe] contribute actively to the realisation of this project," the statement concludes.

Turning that enthusiasm into funding will be a challenge, however. Elsen believes that CERN would be the logical organization to coordinate European participation in the ILC. But the priority at CERN is an LHC upgrade. "Whether new funding can be obtained from the European Union or individual European countries is a matter of negotiations—and of financial resources," Elsen says. Harrison says the U.S. budget for high-energy physics "would need to be increased should the ILC proceed and the U.S. participate." And he says "it is less than obvious" whether that funding could be found in time for a 2015 start.

A long delay could affect the project's scientific value. Yamashita says physicists would like to see the ILC turn on while the LHC is still operating to take advantage of complementary capabilities. Harrison says that complementarity also means that the ILC would not be irrelevant in the foreseeable future. But he adds, "10 years from now it might not be the right thing to start."

—DENNIS NORMILE

With reporting by Adrian Cho.

BIOMEDICAL CAREERS

NIH Offers to Help Universities Improve Training, Boost Diversity

For 2 decades, some social scientists and biomedical researchers have complained that U.S. universities are training far more graduate students and postdocs than will ever find jobs in academic research. However, others argue that there is no oversupply because these Ph.D.s will find jobs in industry or other sectors. But almost everyone agrees that the current training system takes too long and isn't designed to prepare young biomedical scientists for nonacademic jobs.

Last week, the National Institutes of Health (NIH) announced a plan to prepare scientists for a range of careers, move students through their Ph.D.s faster, and bolster the pay of postdocs. NIH officials say the changes are not aimed at reducing or even stabilizing the number of graduates but are intended to improve the training experience.

NIH is responding to a June report from a working group of the director's advisory committee co-chaired by Princeton University President Shirley Tilghman. The report found that only about 43% of biomedical Ph.D.s trained in the United States will wind up in academia. At the same time, only 2% are unemployed (see graph).

Tilghman, who also co-authored a 1998 National Academies' report on the same topic, believes that NIH is training too many young scientists in a system that she calls "dysfunctional." Her committee's report recommended that the overall number in training should stay the same, however. And last week, NIH decided not to address the question of whether to limit the number of scientists being trained. NIH Deputy Director for Extramural Research Sally Rockey, who co-chaired the June report and is now overseeing its implementation, said that NIH would first need to do a modeling study that would take into account a "huge bolus" of foreign-trained postdocs in U.S. labs.

But NIH agrees that universities need to do a better job of training their students. Toward that end, Rockey says, NIH will fund up to 50 grants over the next 2 years for institutions to develop "innovative approaches" that could include, for example, exposure to industry or science policy positions. NIH will also "encourage" institutions to limit NIH support for graduate students to 5 years, with exceptions for some programs and for fam-

ily leave or if a mentor dies. The agency will increase the number of awards for two small programs aimed at giving young investigators their own labs more quickly.

NIH also intends to require that every graduate student and postdoc supported by NIH work with his or her adviser on an Individual Development Plan that examines career options. "We have to change the culture" so that investigators "are invested in the positive outcomes of their trainees," Rockey said. The agency will also raise its starting postdoc annual stipend from \$39,000 to \$42,000 as soon as next year and examine whether NIH needs a policy to improve postdoc benefits.

Cathee Johnson Phillips, executive director of the National Postdoctoral Association in Washington, D.C., says the NIH plan is "consistent" with recommendations from the group. "These are important steps in helping postdocs to be valued and not just be treated as cheap labor."

NIH also says it wants to fill a gap that stymied the Tilghman working group: a lack of data on where trainees end up. NIH will create a system to track the careers of all trainees, not just those on training grants. Graduate students will receive a unique NIH identification number that is now assigned only to investigators and postdocs.

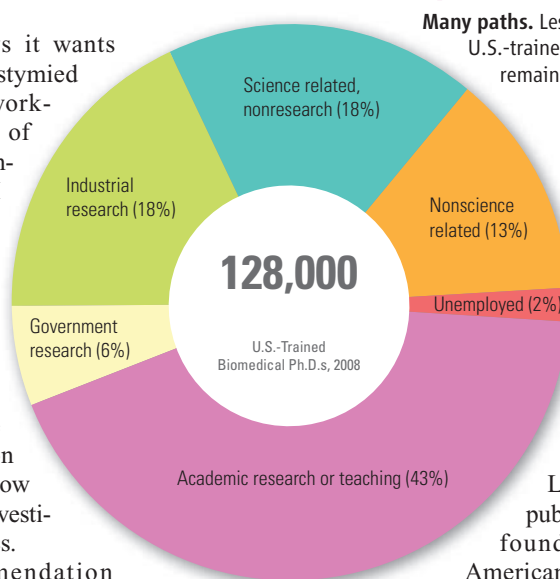
One recommendation from June that NIH is not following is to shift some graduate students and postdocs from research grants, the dominant form of support, to training and fellowship grants, which offer a higher-quality experience. That would have been "extraordinarily complicated to do," Rockey said, partly because NIH's budget separates the two types of programs. However, Rockey says the steps that NIH is taking will accomplish the same goals.

Judith Bond, president of the Federation of American Societies for Experimental Biology, says her group is relieved that NIH is not going to move research funds into training or try to manage the supply side. "I don't think it's at all clear that we need to reduce the number" of new scientists, Bond says. "What's needed is to find ways to use their training for the benefit of society."

Tilghman was cautiously positive about NIH's plan. "This is the most attention manpower issues have received at NIH in my career," she said. But she also told the advisory committee about her "cynicism" that institutions will follow through because many of the changes are voluntary. "Without the financial pressure to get people through in an expeditious length of time, I think we will be looking at data that looks just like this 10 years from now."

NIH Director Francis Collins acknowledged Tilghman's concern that the plan lacks "real teeth." He and Rockey suggested that NIH could not force institutions to track all graduates but that perhaps such rules could be applied to new traineeships.

Where They Work



Many paths. Less than half of U.S.-trained biomedical Ph.D.s remain in academia.

At the same meeting, NIH officials said they plan to spend roughly \$50 million a year over 10 years to increase the diversity of the biomedical workforce.

Last year, a study published in *Science* found that African American researchers are much less likely to receive grant funding from NIH than white scientists with a similar research record.

Most of the new money, added to the \$135 million that NIH now spends, will fund scholarships and research experiences for minority college students. NIH is also launching a mentoring network to help minorities craft strong grant proposals and will test the use of anonymous grant applications to address possible bias in peer review.

—JOCELYN KAISER

Turing Pattern Fingered for Digit Formation

Born in 1912 in London, Alan Turing has been the subject of numerous centenary celebrations by scientists around the world this year. The mathematician, known as the father of computer science and artificial intelligence, is still prompting new discoveries more than 50 years after his death. On page 1476, a team of biologists offers fresh evidence that one of Turing's last theories guides how some parts of the body develop.

The new work concerns the mystery of how mammalian limbs get their standard five digits. The suite of genes that governs limb development includes some of the best-studied in the field, but explaining why a hand has five fingers—and why some mutations cause more or fewer to form—has left developmental biologists perplexed.

The answer may lie in a set of equations Turing developed. In 1952, 2 years before his untimely death, the man who helped break the German Enigma code during World War II published a paper on “The Chemical Basis of Morphogenesis” that described how two interacting chemicals diffusing through space could form interacting wave patterns that produce spots like a leopard's or stripes like a zebra's. The pattern depends on how fast the molecules diffuse, whether they activate or inhibit each other, and other factors—all parameters of Turing's equations.

The theory has long fascinated theoretical biologists, but it had found less support among those studying actual development. Recently, however, experiments have suggested that Turing mechanisms play a role in the growth of feathers, hair follicles, the branching pattern of lungs, and even the left-right asymmetry that puts the heart on the left side of the chest. Turing's model also appears to describe the pattern that leads to digit formation in the developing mouse paw, concludes the new study by developmental biologists Maria Ros of the University of Cantabria in Santander, Spain, and Marie Kmita of the Clinical Research Institute of Montreal in Canada; theoretical biologist James Sharpe of the European Molecular Biology Laboratory Centre for Genomic Regulation in Barcelona, Spain; and their colleagues. The work is a significant advance for Turing-type mechanisms in development, says Shigeru Kondo, a theoretical biologist at Nagoya University

in Japan, especially because limb formation commands more respect among developmental biologists than pigment patterns or feather buds.

A widely accepted model of digit formation involves the diffusion of proteins and signaling molecules to form a relatively smooth gradient across the developing hand or paw. This gradient, some argue, contains enough information to tell a cell whether it should condense into cartilage and form the beginnings of a finger or toe or ultimately die back, forming the space between digits.



Revealing paws. As more *Hox* genes are removed from a developing mouse limb lacking the gene *Gli3*, more digits form, a pattern fitting a model developed by mathematician Alan Turing.

Ros and Kmita were exploring the process of digit formation by studying *Hox* genes. The subject of the 1995 Nobel Prize in physiology or medicine, the *Hox* family of genes famously helps control the head-to-tail patterning of embryos. Previous studies had shown that certain *Hox* genes interact with genes called *Shh* and *Gli3* as the mouse paw is forming, and that knocking out either *Shh* or *Gli3* causes extra digits to form—typically seven or eight. One theory was that removing those genes led to a higher-than-normal dose of the *Hox* genes, which then caused extra toes. Ros and Kmita thought eliminating the

activity of some of the *Hox* genes could bring the digit number back down to five.

What they saw was the opposite. When they knocked out various *Hox* genes in addition to knocking out *Gli3*, the resulting mice had even more digits. And the more *Hox* genes they removed, the more digits formed. The most extreme mutants had 14 toes on their developing paws.

The researchers were surprised by another observation: As they took away more *Hox* genes, the number of digits increased, but the overall paw size remained relatively unchanged. Other experiments had suggested that additional digits resulted from more tissue. But the thinner and more densely packed digits suggested that losing *Hox* genes seemed to shorten the spacing between digits—the wavelength in Turing's mathematical language.

Theoreticians have suggested for decades that a Turing-type wave function might help pattern fingers and other digits, Sharpe says. But, he adds, the 14-toed mice and other *Gli3/Hox* knockouts are the first example of a genetic modulator of wavelength—one of the missing pieces of evidence that Turing's theory could be at work in hand development.

Several key pieces are still missing, however. Turing's model requires two diffusing and interacting molecules that form a pattern, and *Hox* genes don't diffuse through tissue. Instead, Sharpe says, they must control other—still mysterious—factors that signal which cells start to form cartilage—and ultimately digits. “The *Hox* genes are known to be desperately important in limb development, but it has been almost impossible to name tangible roles for them,” he says. “In this phase of limb development, their role seems to be to regulate the Turing mechanism.”

A Turing “model fits quite well,” says Denis Duboule, a developmental biologist at the Swiss Federal Institute of Technology in Lausanne, though he says he'd like to see if an experiment that increases *Hox* genes results in the formation of fewer digits. “The next question is, how does it work?” *Hox* genes are transcription factors that turn other genes on and off, Duboule notes, so they influence multiple genetic pathways. “I must say, it's a very difficult question.” —GRETCHEN VOGEL

Drilled. An outside panel says a report on fracking's health and environmental risks was badly flawed.



ENERGY RESEARCH

University of Texas Revamps Conflict Rules After Critical Review

It's rare to see the accused and the accusers in a conflict-of-interest case end up on the same note. But that appears to be what happened last week when the University of Texas (UT), Austin, accepted an independent review panel's harsh findings, echoing outside critics, about a controversial study conducted earlier this year by UT's Energy Institute. The topic: hydraulic fracturing for recovery of natural gas, or "fracking." Among the consequences of the scathing review, UT officials say, is that the university is revamping its conflict-of-interest policies and looking for new institute leaders.

Critics charged last summer that the UT report—which examined the human health and environmental implications of fracking—was tilted in favor of the gas industry. And they revealed that its principal manager—former U.S. Geological Survey chief Charles Groat—had an undisclosed financial stake in a gas exploration company. In August, UT commissioned an outside panel to take a look. That three-member group—chaired by retired aerospace executive Norman Augustine, a former council chair of the National Academy of Engineering—handed UT 33 pages of findings on 30 November, essentially supporting the critics' views.

On 6 December, UT Austin Provost Steven Leslie made the review public and said that the university had accepted all of its findings. UT had already started to overhaul conflict and research management before the furor, he said: These steps will be "extended" and implemented as soon as possible. In addition, Leslie said, the controversial fracking

report has been withdrawn from their Web site, and UT faculty and staff members have been asked not to discuss it until it undergoes a complete scientific review. Groat, meanwhile, has retired from the university, and Energy Institute Director Raymond Orbach, a former research chief at the U.S. Department of Energy, stepped down from his post on 30 November. Orbach, who declined to comment, remains on the UT faculty.

The 414-page fracking study, titled "Fact-Based Regulation for Environmental Protection in Shale Gas Development," grew out of an effort by Groat and the Energy Institute to produce a major scientific study on fracking with funding from the American National Gas Association, according to the Augustine review. But the partnership fell apart, the review says, because industry officials "insisted upon managing the project in detail, including removing one of the investigators and editing the report prior to its release."

After withdrawing from the partnership, Groat obtained internal university funding for the study—a "commendable" move says the review, which was co-authored by former National Science Foundation Director Rita Colwell and president emeritus of the University of Michigan James Duderstadt. But the scope of the report shrank, it says, and quality control appeared to falter. Those problems began to surface after Groat presented a draft version of the report to the media at the February meeting of AAAS (publisher of *Science*).

Although many press accounts reported that the study concluded that fracking posed little threat, the nonprofit Public Account-

ability Initiative (PAI) in Buffalo, New York, was skeptical. In July, the group released a harsh critique that concluded that the study presented no new science but minimized fracking's risks through selective presentation of data. And when PAI went looking for undisclosed financial conflicts, it found stock proxy statements showing that Groat was on the board of the Plains Exploration & Production Co. of Houston, Texas, which uses fracking to extract gas, and that Groat had nearly \$2 million equity in the firm. The group estimates that Groat's income from the company was more than twice his UT salary.

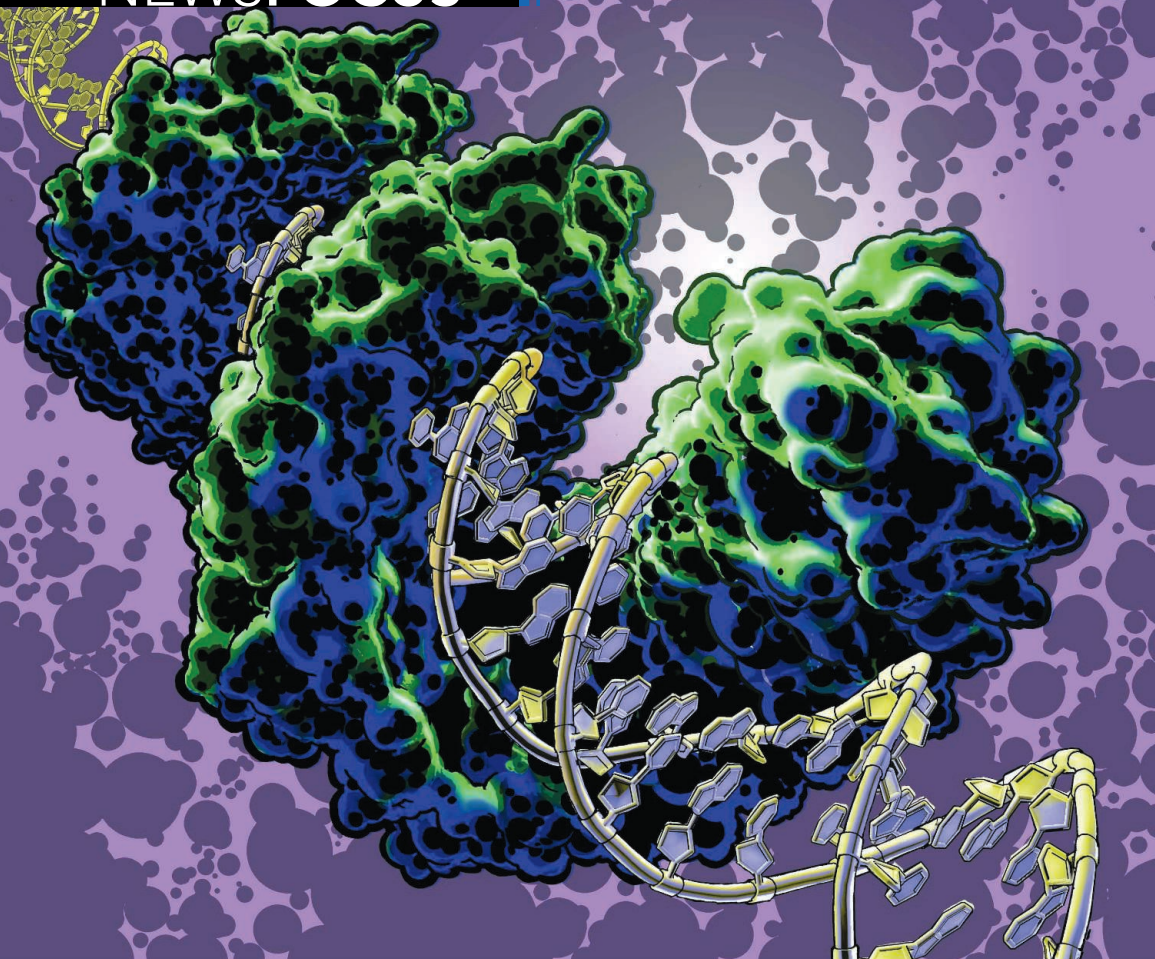
The outside review supports many of PAI's complaints. The final version of the report had many flaws, it said, including a summary that appeared to be pushing the message that "there was little need for further regulation" of fracking. Papers by the senior contributors, the reviewers found, "were not subjected to serious peer review," and the project was "hampered by the absence of knowledgeable senior leadership."

The review panel also singled out Groat's failure to disclose a potential conflict of interest as "[p]rimary among the shortcomings." The reviewers found "no evidence of intentional misrepresentation," or that Groat's board membership shaped the report's findings. But his failure to disclose his industry ties reflected "very poor judgment coupled with inattentiveness." The reviewers noted, however, that Groat was "probably not in violation of the University's Conflict of Interest Policy as it existed" at the time, because it applied only to external, not university, funding for the study.

"In hindsight, I should have disclosed my board membership," Groat wrote in an e-mail to *Science*. "I regret that this oversight has understandably caused concern regarding the study." Groat is now president of The Water Institute of the Gulf, a nonprofit research organization based in Baton Rouge.

UT's Leslie said he was "quite surprised" by what the Augustine panel wrote. He had expected a bad grade on the disclosure issue, not for problems in research. "We thought the review would find a strong research project. But that was not the case."

Report critic Kevin Connor, director of PAI, says that he, too, was surprised by the panel's findings. The PAI report was tough, he says, but the Augustine review is "far more damning." It surprised him partly because he knew Augustine has connections to the oil industry (he served on the board of ConocoPhillips, for example). Connor says: "I applaud the fact that UT has put it out and agreed to the findings." —ELIOT MARSHALL



Spot on. In this cartoon rendering, a TALE protein homes in on a specific DNA target.

Companies and academics have since come up with ways to make these proteins cheaply and easily, making them accessible to an ever-broader range of researchers. Engineered TALEs have now targeted a wide range of genes in a variety of organisms. At least one biomedical team wants to harness the technology to treat human disease, specifically sickle cell anemia. "In several years, it might be possible to modify crops in a very targeted way or cure disease," Boch says. "It's a game-changing tool."

TALEs face competition, however. A related genome-engineering technology involving proteins called zinc finger nucleases has a 15-year head start and is in clinical trials. "For an academic, TALEs represent an easier platform to get started with, but that doesn't necessarily make it the

best platform for therapeutics," says Philip Gregory, chief scientific officer at Sangamo BioSciences in Richmond, California, which is developing zinc finger nucleases for biomedical applications. In addition, just as TALEs have rushed into the limelight, another gene-targeting strategy, based on strands of RNA rather than proteins, is poised to prove its worth (see sidebar, p. 1411).

Pioneered in plants

Genome engineering was not exactly what Boch had in mind in 2007 when he was trying to figure out how the plant pathogen *Xanthomonas* does its dirty work. Different versions of this bacterium attack more than 350 plant species, including major crops and fruit and nut trees, causing diseases such as citrus canker and black rot. At Boch's university, Ulla Bonas, Thomas Lahaye, and colleagues had shown that a *Xanthomonas* protein—the first of a group of proteins later named TALEs—enters the nucleus of pepper cells and takes control of a gene that regulates cell size, causing plant cells to grow extra large (*Science*, 26 October 2007, p. 648). Those researchers were looking to connect other TALE virulence proteins to their respective target genes.

The Tale of the TALEs

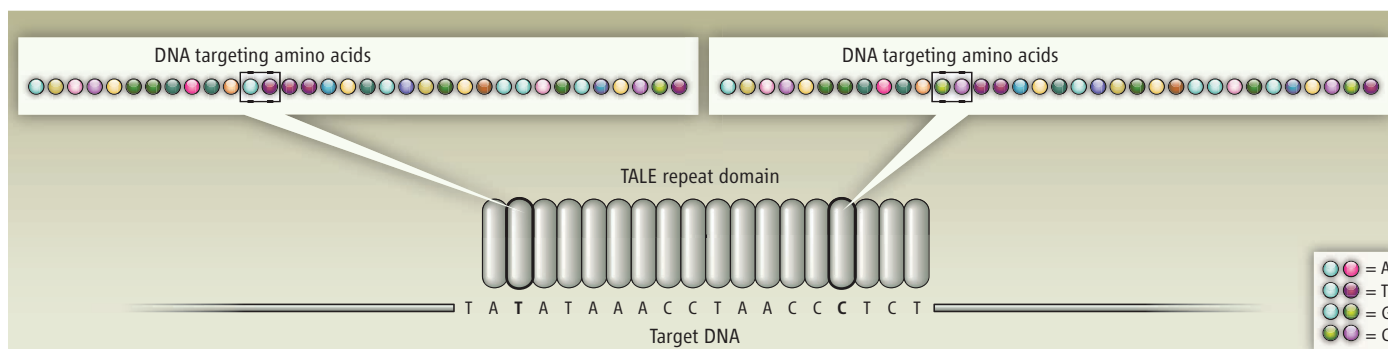
Biologists have turned plant pest proteins into tools for studying and reshaping genomes of many species

SOME OF BIOLOGY'S BEST TECHNOLOGIES come from unexpected places. The green fluorescent protein that lit up biology with its ability to track proteins and gene expression in cells was borrowed from a jellyfish. A heat-stable enzyme from a bacterium often found in hot springs made the polymerase chain reaction method practical, facilitating the easy copying of DNA fragments needed for a myriad of applications, including the DNA fingerprinting used so widely to identify people. Now, thanks in part to inspiration that struck during a lunchtime discussion, proteins from a feared plant pest are poised to make genome engineering, the large-scale, directed manipulation of genes, routine for researchers studying a variety of organisms, including yeast and humans.

Like cruise missiles attacking military targets, these proteins, called transcription activator–like effectors (TALEs), can be

programmed to home in on specific DNA sequences and carry out an action once there. When attached to enzymes called nucleases that cut DNA, for example, they can knock out a gene or change its sequence. TALEs "are one of the hottest topics in genome engineering," says Jens Boch, the plant pathologist at Martin Luther University Halle-Wittenberg in Germany who was one of the first to show the potential of these proteins.

Five years ago, researchers were just figuring out what TALE proteins do in plants. Then in 2009, two back-to-back papers in *Science*—one by Boch's team—solved how these proteins target specific genes, demonstrating a simple code that, in theory, could be applied to home in on any set of DNA bases (11 December 2009, p. 1501 and p. 1509). In the next year, the first nucleases were attached to these proteins, revealing their potential for genome engineering.



One-to-one code. Each TALE repeat targets a specific DNA base: A, T, C, or G. The repeats are the same series of 34 amino acids (color-coded) except at positions 12 and 13, which differ depending on the base being targeted.

Over lunch in their office, Boch and colleague Sebastian Schornack pondered the unusual structure of the TALEs. Large sections of these virulence proteins consist of multiple sets of the same, or almost the same, 34 amino acids occurring in tandem. Boch and his colleagues wondered if these repeats somehow specified a TALE's DNA target. Using the sequence of the regulatory element of the cell-size gene and its associated TALE to break the code, they predicted the targets of other TALEs, verifying them in further experiments.

The key was the variable pair of amino acids in the middle of each repeat. (The rest of the 34 amino acids are nearly always the same.) What Boch and his colleagues discovered was that if the pair consists of histidine followed by aspartic acid, then that repeat targets the DNA base cytosine. An asparagine and a glycine at those spots, however, will cause the repeat to recognize the base thymine. The other two DNA bases—adenine and guanine—are similarly targeted, and the overall number of repeats matches an equal number of bases. A 17-repeat TALE homes in on a specific 17-base stretch of DNA, for example. At the time, Boch recalls, "it was a very bold idea that one repeat would correspond to one base."

Yet, in the spring of 2009, just as Boch was putting the finishing touches on work demonstrating the existence of this code, his colleague Bonas got an e-mail from Adam Bogdanove, then at Iowa State University in Ames. While looking at differences in TALEs between two *Xanthomonas* variants that infect rice, Bogdanove had independently come up with the same bold idea. Working with Iowa State graduate student Matthew J. Moscou, an expert in bioinformatics, he tested his idea on 10 TALEs and broke the code. "We ran the analysis overnight and just bang, it jumped right out," Bogdanove recalls.

The groups' *Science* papers initially garnered mixed reactions, however. "When I first heard about this, I was skeptical that something so simple would work for DNA binding proteins," says J. Keith Joung, a molecular biologist at Massachusetts General Hospital in Boston who worked extensively with zinc fingers. "I made a conscious decision to just watch."

But Sangamo's Gregory was hooked, even though TALEs may compete with Sangamo's zinc finger technology. "The elegance of the biology here was just captivating," he recalls. Like TALEs, zinc fingers—fingerlike sections of certain DNA binding proteins found in mammals—home in on specific target sequences of DNA (*Science*, 23 December 2005, p. 1894). Different zinc fingers recognize different sets of three bases, and by combining various zinc fingers into an engineered protein, researchers had shown that they could target specific DNA sequences. Sangamo had harnessed this specificity by linking nucleases to sets of zinc fingers, and over the past few years, researchers have used zinc finger-endowed enzymes to knock out genes and

even to introduce new versions of genes at specific locations in the genome, something that was almost impossible to do before.

While increasingly popular, zinc fingers have their issues. "The problem with zinc finger nucleases is that they are difficult to design and could be expensive," says Rudolf Jaenisch, a developmental biologist at the Whitehead Institute for Biomedical Research in Cambridge, Massachusetts, who uses such nucleases to modify human embryonic stem (ES) and induced pluripotent stem (iPS) cells. One explanation for the high costs is that Sangamo, to the consternation of many scientists, controls almost all of the intellectual property rights to zinc finger technology and kept a tight grip on their production and use for a while. TALEs could provide a cheaper alternative.

Developing TALENs

Once he had the code in hand, Bogdanove immediately wondered if TALEs could replace zinc fingers as the targeting mechanism for nucleases—creating so-called TALENs. He asked Iowa State plant biologist Daniel Voytas for help. A zinc finger enthusiast, Voytas had, with Joung, spent several years making that nuclease technology more available to academics, and he wanted to use it for improving and studying plants. Yet Voytas, too, was at first hesitant about the one-to-one TALE code. "It seemed too easy," he recalls.

But by 2010, Voytas, now at the University of Minnesota, Twin Cities; Bogdanove; and their colleagues had found that they could attach nucleases to TALEs. Another group at Iowa State led by Bing Yang independently had the same success that year, and both groups published demonstrations of TALENs cutting specific DNA targets in yeast.

The following year, the most convincing evidence of the poten-



Big-hearted. A swollen heart cavity (arrow) in the TALENs-treated zebrafish (top) results from knocking out a specific gene.

tially broad utility of TALENs came from Sangamo. Jeffrey Miller and colleagues at the firm trimmed off the ends of TALE proteins, so that they were fusing streamlined versions with a nuclease. These TALENs worked in mammalian cells and could be used just like zinc finger nucleases to knock out or put in a new gene, the Sangamo team reported in the February 2011 issue of *Nature Biotechnology*. “This publication ignited the push to use TALENs,” says Veit Hornung, an innate immunologist at the University of Bonn in Germany.

Joung and his colleagues jumped on board, testing TALENs in zebrafish, a model organism useful for many kinds of biological studies. They, and, independently, a team from China, reported in the August 2011 issue of *Nature Biotechnology* that specific genes could be knocked out by putting appropriately targeted TALENs into zebrafish embryos. “Everything we made worked—that was not so with zinc fingers,” Joung recalls.

Wondering whether his team had just gotten lucky, Joung and his colleagues developed a high-throughput, automated method of making TALEs. Called FLASH, the method has begun to turn TALEs into a commodity, Joung says, “the cost of which will continue to come down.”

In one follow-up experiment using 144 FLASH-made TALENs, Joung confirmed his group’s earlier, surprising success rate. Compared with a typical success rate of 50% in publicly available zinc finger nucleases, “90% or more are active in human cells,” says Joung, who described FLASH and these results in the May issue of *Nature Biotechnology*. “It changes the way you think about DNA binding technology.”

And unlike zinc finger nucleases, Sangamo doesn’t have a lock on TALENs or other TALE-equipped tools. No patents have been issued yet in the field, and already one can buy TALEs and TALENs from at least two companies—Life Technologies in Carlsbad, California, or Collectis, in Paris—or get kits to make them in one’s own lab using different methods. (The nonprofit organization Addgene has distributed 824 kits and is now averaging about 50 per month, Voytas says.)

At a September meeting on genome engineering held in Italy, Bonn’s Hornung described yet another fast, cheap way of

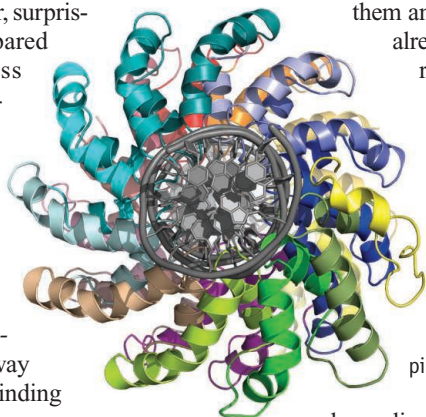


Made to order. Researchers used TALENs to deprive this pig of a lipid receptor, making it useful for studying heart disease.

making TALEs, one that has already yielded 1500 TALENs. He plans eventually to make a TALEN to target every human gene. “You can produce a lot of TALENs in a very short time, which will allow you to ask questions you didn’t dare ask before,” says Toni Cathomen, a molecular biologist at the University Medical Center Freiburg in Germany.

Applying TALENs

Whitehead’s Jaenisch is one of many converts. He originally used zinc finger nucleases to modify disease-related genes in ES and iPS cells, but with Sangamo’s help, his team recently did a direct comparison between them and TALENs, targeting five already-studied genes or gene regions. The results were comparable and to date, he and his lab have used TALENs against a dozen genes in ES and iPS cells and “have not



Close fit. As they latch on to DNA, TALE repeat domains flare out to form a pinwheel structure.

been disappointed,” Jaenisch says. Because of the ease in making TALENs, “the zinc fingers we use less and less,” he adds.

Thierry VandenDriessche, a molecular biologist and gene therapy expert at the Free University of Brussels says that’s a trend: “Almost everybody who has worked on zinc fingers is shifting now to TALENs.”

Indeed, in the past 2 years, researchers using TALENs have engineered genomes in nematodes, tobacco, the model plant *Arabidopsis*, rats, crickets, livestock, and zebrafish. In the latter case, reported in the 1 November issue of *Nature*, Stephen Ekker, a molecular biologist at the Mayo Clinic in Rochester,

Minnesota, led a group that knocked out zebrafish genes much more efficiently than had been done before and proceeded to observe the resulting defects as the fish embryos developed. In some experiments, the deleted portion of the gene was replaced with other DNA, demonstrating TALENs’ potential for precise gene modification in these animals. One of the sequences added will enable Ekker’s group to switch a gene on and off, so they can see the gene’s role at different points in development.

TALENs have also been a welcome addition to the toolbox of those seeking to genetically modify pigs, cows, and other livestock to make versions that are more useful for biomedical research or food production. Scott Fahrenkrug, CEO of Recombinetics in St. Paul, Minnesota, notes that his firm had had limited success using zinc finger nucleases for genome engineering of livestock. But about 65% of the TALENs tried in embryos or cells taken from live pigs and cows have worked, some very well, he says. His firm has already used TALENs in miniature pigs to knock out their gene for the low-density lipoprotein receptor, creating a pig strain that is potentially useful in the study of cholesterol-related diseases. Fahrenkrug and his colleagues reported that result in the 23 October issue of the *Proceedings of the National Academy of Sciences*, and in unpublished studies, they have introduced new DNA into cow cells via TALENs.

Armed with that skill, Recombinetics is currently trying to get financial support to create a breed of dairy cattle that carries a beef cattle version of a gene that is important for horn development. Dairy cattle have to have their horns removed, but with this version of the gene, they would never develop horns to begin with. It’s a way of doing “precision crossbreeding,” Fahrenkrug says, that can greatly speed up the introduction of new traits to livestock.

Others are looking at TALENs with an eye toward gene therapy. Huimin Zhao, a bioengineer from the University of Illinois, Urbana-Champaign, is one such researcher. In the April issue of *Molecular BioSystems*, his team demonstrated in yeast that a TALEN can correct the genetic defect underlying sickle cell disease. Unpublished studies, he adds, show that corrections can be made in cells taken from sickle cell disease patients as well. Using TALENs, “there are many genetic diseases that could be targeted,” Zhao says.

A few researchers are working on additional applications for the DNA-targeting aspects of TALEs. Some have been designing TALEs that will go into a cell and turn on a specific gene, giving finer control over gene expression. Studies have shown that these designer TALEs work in both plant and human cells and can increase gene expression 20-fold or more. Others have made TALE proteins that repress genes.

Robert McKnight, a molecular biologist at the University of Utah in Salt Lake City who is working to link TALEs to enzymes that add methyl groups to DNA. Such methylation of DNA typically shuts down the associated gene. By precisely methylating certain DNA sequences, McKnight also hopes to gain a better handle on how such biochemical modifications influence genome function, a field commonly known as epigenetics.

As for the plant pathologists who first figured out the TALE code, they are going full speed ahead. Boch is hoping to use customized TALENs to thwart the natural TALEs made by the plant pathogens he has long studied. With support from the Bill & Melinda Gates Foundation, he and French collaborators plan to knock out the DNA in rice to which *Xanthomonas* TALEs attach, which should reduce the damage caused by an infection. There is some indication that regulatory agencies will not treat these TALE-engineered crops as genetically modified organisms—TALENs and zinc finger nucleases are simply mutagens, some argue—and that could pave the way for their easier adoption.

Bogdanove's emphasis has expanded. "We sort of have a split personality," he explains, with some work on plant pathology and other efforts aimed at "the basic biology of the proteins"—including helping to determine the molecular structure of a TALE-DNA complex. (A second group did likewise at the same time; *Science*, 10 February, p. 716 and p. 720.) His lab has also engineered a rice plant so that genes that counter infection are turned on by TALEs.

Not perfect

The road to practical use of TALENs could still contain potholes. TALENs are bigger and bulkier than zinc finger nucleases, so getting them to their DNA targets may sometimes be tricky. Also, the

Beyond TALENs

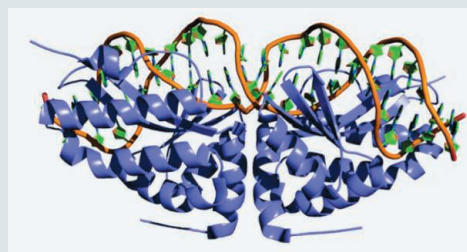
Zinc finger nucleases and TALENs are not the only up-and-coming genome engineering tools.

Meganucleases are DNA-cutting enzymes encoded by certain mobile elements, DNA sequences that hop from one chromosomal location to another. They recognize short DNA sequences and can be modified to bind to different DNA targets. But doing so is tricky because those changes also affect the enzyme's cutting ability, says J. Keith Joung, a molecular biologist at Massachusetts General Hospital in Boston. One solution is to combine a meganuclease with a TALE.

And right now, TALEs have to work in pairs, because two nucleases have to come together in order to cut DNA. A few researchers are trying out nucleases that can do the job solo.

Another idea in the works entails harnessing a bacterial defense system that involves nucleases attached to RNA. The sequence of the RNA determines the target. Called CRISPR, the system has potential for providing DNA targeting using RNA instead of TALEs or zinc finger proteins, Emmanuelle Charpentier from Umeå University in Sweden and colleagues reported in the 17 August issue of *Science* (p. 816). If this approach pans out, it could supplant both zinc finger nucleases and TALENs because of the ease of using RNA over proteins.

—E.P.



DNA slicer. A meganuclease (purple) with DNA.

repetitive nature of a TALEN means that the gene encoding it, once delivered into a cell, may be more susceptible to DNA rearrangements that compromise the enzyme's actions.

Gregory also worries that TALENs will have so-called off-target effects, where the nuclease cuts unintended DNA sequences. While these effects have thus far proved minimal with TALENs, they also didn't seem to be an issue in the early days of zinc finger nucleases either, but have since been recognized as a problem in some.

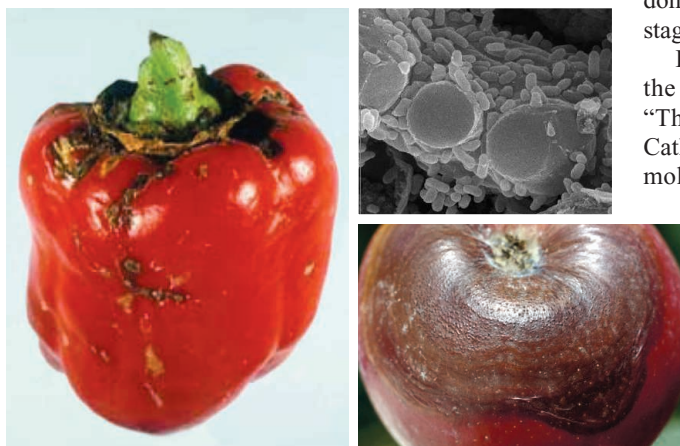
And when it comes to modifying a gene's code, rather than just knocking out a gene, both zinc finger nucleases and TALENs still need improvement, researchers stress. "I am not sure there's going to be a simple

answer, where one platform is always better than the others," Joung says. "It will depend on the application."

For its part, Sangamo says it will continue to push zinc finger technology while also continuing to look at TALE-based tools. Initial, ongoing clinical trials are already testing the potential to cure HIV infections by using zinc finger nucleases to knock out the gene for the T cell receptor that the virus uses to get into the cell. At the Society for Neuroscience meeting in New Orleans this October, Sangamo scientists also described progress using zinc finger technology to knock out just the mutant version of the Huntington's disease gene, while leaving the other, normal copy of the gene intact. "We don't see a need to switch to TALENs at this stage," Gregory says.

But to others, particularly academics, the potential of TALEs seems limitless. "The growth we have seen is enormous," Cathomen says. Basic biologists can study molecular pathways gene by gene, protein by protein. Plant biologists will be able to introduce multiple traits at once, greatly speeding up the time it takes to develop disease-resistant strains. "It will just become a tool that every molecular biologist has in the lab," Cathomen adds. In the coming years, "we will see an explosion of results using TALE nucleases in many different areas of biology."

—ELIZABETH PENNISI



Plant menace. Peppers and apples are among the many crops destroyed by *Xanthomonas* (top right) bacteria.



Rock 'n' roll. Gray concrete "smart rocks" and colorful radio-tagged "tracer rocks" await a bumpy ride.

GEOMORPHOLOGY

How to Build a Smarter Rock

Predicting when and where rivers will move gigatons of rock and sediment has proved a murky problem; a new generation of electronic smart rocks could clarify matters

In April 2011, one of the largest and longest floods on record coursed down Reynolds Creek, a steep mountain stream that runs through sagebrush meadows and aspen groves in southern Idaho. Fed by snowmelt from the Owyhee Mountains, the floodwaters tore down river banks and sent a jumble of rocks tumbling downstream.

Some of them were no ordinary pebbles. More than 200 were brightly painted natural rocks that contained radio tags inserted into specially drilled holes. Four others were "smart rocks" made of sleek brushed aluminum. Researchers had crafted each metal rock to mimic a natural stone's shape and density, and then inserted custom-made electronics that could measure and record movements 512 times per second. It was the smart rocks' first trip down a real river; previously, they'd been coddled in a carefully controlled laboratory river called a flume. Their mission: to help researchers better understand how waterways move tons of rock and other sediment downstream.

It is no small issue. Worldwide, rivers transport an estimated 13 gigatons of sediment each year, more than any other force on Earth besides humans. But predicting where that material will end up has proved difficult. Current models for predicting the movement of coarse sediment in a river—some based on equations developed by Albert Einstein's son Hans in the 1950s—are frequently off by at least an order of magnitude, says Joel Johnson, a geomorphologist at the University of Texas (UT), Austin. That's a problem

for engineers trying to protect bridges, dams, and levees from shifting flows, and ecologists trying to plan expensive river restoration projects. Researchers, meanwhile, have struggled with the difficult and dangerous task of finding out what's really going on during floods, when rivers do most of their heavy lifting. That's why Johnson and other researchers are pioneering a new approach: building increasingly sophisticated smart rocks that are intelligent enough to take measurements on their own. The devices, Johnson says, are "a killer app" that gives scientists a unique glimpse of river dynamics "from the point of view of the rocks."

Chasing marbles

Deploying objects to track shifting river sediments is not a new idea. In the 1960s, the influential late geomorphologist Gordon



Not your average rock. Custom-made aluminum rocks hold sophisticated electronics that help researchers pinpoint forces that send them tumbling.

"Reds" Wolman of Johns Hopkins University in Baltimore, Maryland, dropped marked marbles into a nearby stream, then noted how far they moved. He continued to find the marbles into the 1980s; he even offered students six beers for every marble recovered, recalls fluvial geomorphologist Peter Wilcock of Johns Hopkins. Decades later, he says, one student found a stash, but "I don't believe they ever received the payout. ... The link to immortality was presumably sufficient compensation."

Since then, researchers have tried to make it easier to find such tracers by using rocks embedded with iron magnets or even radio-frequency identification tags similar to those used to identify lost pets and track merchandise. Those tracers, however, can reveal only how far an object travels, not fine-scale information about the forces that set rocks tumbling or what happens along the way. "We know the rocks go downstream—we're not idiots," says Joanna Curran, a hydrologist at the University of Virginia in Charlottesville. But because sediment transport is a nonlinear physical process, small mistakes in input measurements can result in disproportionately large output errors in mathematical model predictions. Improving the models means getting down to nitty-gritty details, including better measurements of dozens of variables ranging from large-scale channel slopes and water velocities to minute interactions between a single grain of sand, the water flowing around it, and the river bed. Sediment scientists, Curran says, want to measure forces down to the level of a rock's "skin."

Now, advances in materials and electronics are making that possible. In one project, Curran is helping undergraduate students use a three-dimensional printer to make plastic smart rocks about 7.5 cm across. Each has

CREDITS (TOP TO BOTTOM): LINDSAY OLINDE, UNIVERSITY OF TEXAS, AUSTIN, DEPARTMENT OF GEOSCIENCES; JOEL JOHNSON, UNIVERSITY OF TEXAS, AUSTIN, DEPARTMENT OF GEOSCIENCES

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sensors on six sides and a gyroscope that measures the rock's orientation. The gear is helping Curran gain insight into a key river variable: shear stress, the same side-slipping force that tectonic plates produce as they slide past one another, or if you run your hand along a brick wall. Rocks of different sizes and shapes respond differently to the shear stress created by flowing water, Curran says. It takes more shear stress to move an angular rock than a rounded one, for example. To quantify such differences, Curran has been dropping her fake stones into a flume in her lab. They transmit data to a computer through plastic tubes attached to the rocks. Eventually, she'd love to untether her rocks and release them into a real river—to better understand, for example, the best way to take down a dam and restore natural sediment flows. But she's worried about the cost: At roughly \$250 apiece, "these are expensive little things" and she's afraid to lose them.

A rocky start

Such fears, however, didn't stop UT's Johnson from leaving his four aluminum rocks—which cost roughly \$800 apiece—at the mercy of a raging Reynolds Creek early last year. After putting his creations in the remote stream, he and doctoral student Lindsay Olinde waited for the floodwaters to rise and then fall as the snowpack petered out. Then, in July 2011, Olinde hiked back in with a field assistant and began searching for both the hundreds of radio-tagged stones—which cost only about \$5 apiece, not counting labor—and Johnson's four metal mimics. A previous study had suggested that most faux-rocks wouldn't move more than 100 meters downstream. After a week of searching with an antenna that chirped in response to the painted, radio-tagged rocks, Olinde had found only one within the 100-meter reach. "I thought my equipment was broken," she says.

That was just the beginning. Over the next 5 months, she took four more rock-hunting trips, sometimes scrambling through the steep canyons. By November, her gloves were freezing to the icy boulders, and she was hearing the chirp of the radio tags in her sleep. Ultimately, she located roughly 150 of the radio-tagged stones. One-half had moved more than 2500 meters downstream, and a few had tumbled more than 6440 meters. "We had fist-sized particles move almost 7 kilometers," Johnson says, in awe.

Olinde had a similar struggle finding the four aluminum rocks. The chirping radio tags do not work in close proximity to aluminum, so Olinde had to use a metal detec-

tor instead. But the creek turned out to be full of metal objects such as old ranching equipment, making the detector all but useless. Finally, a rancher's dog found the first smart rock in September, more than 2000 meters downstream from where it had been deposited. Olinde spotted a second by accident in November, where it lay in an icy pool 900 meters downstream, glinting in the sun. The other two are still missing in action.

Back at the laboratory, the researchers were relieved to find that the two survivors had collected data despite their rough rides. But relief turned to disappointment when they discovered that the batteries—supposedly strong enough to survive at least a month—had died after just 40 hours. During that brief period, the smart rocks very accurately recorded no movements whatsoever, Johnson says. "We are certain that those rocks stayed still," he says ruefully. "In hindsight, we should have done a lot more testing. ... I was banging my head against a wall."

All was not lost. By combining data on the radio-tagged, natural tracer rocks' unusual distribution with geographic information system data on local topography, Olinde and Johnson are studying how variables such as the steepness and width of the creek channel influenced where the tracers ended up.

Brains vs. brawn

Inspired by last year's experiment, Olinde has been testing a new kind of smart rock along Reynolds Creek. It is somewhat cruder than Johnson's aluminum models, but cheaper and sturdier. She makes them by filling rubber molds of natural rocks with wet concrete, and then inserting a \$100 accelerometer about the size of a matchbox. Its penny-sized battery lasts for months, allowing the accelerometer to record the rock's spatial orientation along three axes every 15 minutes. At that rate, Olinde can't see how moment-to-moment forces influence movement, but she can see how the rocks shift in concert with changing water levels. After inserting radio tags into the rocks, she spray-paints them with neon colors. (The results, she jokes, look like artworks created by a cross between British landscape artist Andy Goldsworthy and the American pop-surrealist Andy Warhol.)

Earlier this year, Olinde released 73 of her new "not-so-smart" rocks into Reynolds Creek, along with 1200 simpler, radio-tagged versions. When the spring floods came, anten-

nae she had installed along the river tracked the rocks as they rolled by. Later, she and a team of assistants searched a 10 km stretch of creek and recovered 33 of the 73 more sophisticated sensors. Unlike Johnson's aluminum rocks, however, the majority of Olinde's concrete versions had continued to collect data throughout their journey. "Joel's rocks are the fine Renaissance gentlemen," she jokes. "My rocks are the burly mountain men."

Olinde is still analyzing the data, but one thing is clear: Current sediment transport models don't do a good job of predicting the rocks' rests and motions. Other researchers



Before the flood. Geomorphologist Lindsay Olinde plunks her faux rocks into Reynolds Creek in Idaho.

agree. The Reynolds Creek work "is a very well done study," Curran says, and "it should add to the body of knowledge on when and why a large cobble moves in a mountain stream." But it also highlights the potential value of scaling up the use of smart rocks to study waterways of all shapes and sizes, from small mountain streams like Reynolds Creek to continent-spanning rivers like the Nile. And it demonstrates the need for even more sophisticated sensors that can reveal the role played by variables like bottom roughness or water depth. "This is the challenge that remains, ... moving from smart to genius rocks," Curran says.

That's a goal Johnson says he'll continue to try to reach. He's working on more robust models of his aluminum rocks and is considering a change in strategy to take into account the rocks' limited battery power: waiting until a flood rises and then tossing the rocks in to record the tumult, even if only for a few hours.

In the meantime, Olinde hopes to help other smart rock researchers avoid problems by writing a methods paper that details the obstacles she encountered and how she overcame them. And she is getting ready to mix another batch of concrete for her sturdy, if less talented, rocks.

—EMILY UNDERWOOD



Expansive view. Christopher Murray led a massive analysis of the “health loss” caused by diseases and injuries.

HEALTH METRICS

A Controversial Close-Up Of Humanity's Health

Kudos and criticism greet a landmark new report, filling the largest ever issue of *The Lancet*, on the global burden of disease

SEATTLE, WASHINGTON—If you had stumbled into Christopher Murray's office in October without knowing who he is or what he does, the cryptic notations written in six shades of felt pen on the whiteboards on his walls would have told a tale as intriguing and revealing as cave paintings. The formulas, graphs, and arrows suggest an ambitious attempt to decipher something exceedingly complex. These are some of the words and symbols scattered about: 187 countries, health, disease, \$, mortality, partnership, methods, and—in bright purple and all uppercase letters—UNCERTAINTY.

Murray heads the Institute for Health Metrics and Evaluation (IHME), a branch of the University of Washington (UW) that contends it has created the most detailed and authoritative report ever on the state of the world's health. The so-called Global Burden of Disease (GBD) 2010 study will appear on 15 December in the largest issue of *The Lancet* ever published, and Murray hopes it will have a major impact on how policymakers, donors, and researchers allocate resources to help people lead healthier, longer lives.

The effort, largely bankrolled by the Bill

& Melinda Gates Foundation, is “a huge, ambitious, and highly disciplined attempt to describe the totality of death and illness in every part of the world,” says global health veteran Richard Feachem of the University of California, San Francisco (UCSF), who chairs an independent scientific oversight group for IHME. “There's nothing else like it or even approaching it.”

GBD 2010 consists of eight papers, 194 pages in total, that examine the epidemiology and loss of health caused by 291 diseases and types of injuries in 187 countries and a whopping 1160 of their lasting effects. It analyzes changes in disability and death from 1990 to 2010; using new computer models based on complex statistics, it ranks the major causes of mortality and morbidity in 20 age groups in 21 regions of the world and identifies 67 underlying risk factors. As Murray's whiteboard telegraphed, the studies give uncertainty intervals for the estimates as well, bringing scientific rigor to a field that often relies on squishy data.

But another type of uncertainty surrounds the project: How much credence will it have with fellow scientists and policy-

makers? Many have questions about how IHME arrived at its results and how they fit with similar efforts by the World Health Organization (WHO), until now the main source of global health data. IHME caused an uproar in February when it gave a sneak peak of GBD 2010 with a paper in *The Lancet* that tallied nearly twice as many malaria deaths as WHO did (*Science*, 15 June, p. 1372). Other numbers may well be equally contentious.

Passions run high about these fights in part because the money spent on research and control measures for any disease is determined largely by the perceived suffering that it causes. Advocacy groups and researchers alike try to trot out evidence that “their” affliction is a major global problem.

In IHME's case, the debates are intensified by some scientists' frustration about what they say is an arrogant attitude and a lack of transparency at the institute. Murray, widely admired for his intellect and abundant enthusiasm and energy, has come under criticism for his domineering style. “There are issues with methods, results, and personalities,” says Dean Jamison, a UW health economist who quit IHME 2 years ago and acknowledges that his views are “clouded by my general lack of perfectly good and cordial relations with Chris Murray.”

This much is certain, however: GBD 2010 demands serious attention. Even its sharpest critics can't ignore it.

Startling patterns

Murray's efforts to take stock of humanity's health go back 2 decades to when the World Bank published a watershed report called *World Development Report 1993: Investing in Health*, prepared by a team that Jamison led. Murray, who has a Ph.D. in international health economics and a medical degree, wrote an appendix that introduced the GBD concept to a wider audience, together with WHO epidemiologist Alan Lopez, who is now at the University of Queensland in Brisbane, Australia.

Until then, the relative importance of diseases had simply been assessed by the number of deaths they caused, which was fairly easy to track. Murray and Lopez wanted to “quantify the full loss of healthy life” and take into account nonfatal conditions such as paralysis, depression, and blindness. They devised a metric called the disability-adjusted life year (DALY), which combined the years of life lost because of a fatal disease or injury with the years of life lived with disability. Controversial at first, DALYs revealed startling patterns. According to the 1993 report, for example, neuro-



Fresher air. Household air pollution from indoor cooking and other sources has decreased over the past 20 years.

psychiatric diseases caused a higher burden worldwide than cancer.

In 1998, Murray moved from Harvard University to WHO's Geneva headquarters to head the Global Programme on Evidence for Health Policy, which created the organization's first burden of disease unit, led by Lopez. GBD reports soon became a mainstay of WHO. Murray returned to Harvard in 2003 hoping to form his own institute, but promised funding fell through; he came to Seattle in 2007 with a \$105 million commitment from the Gates Foundation, which believed that all global health funders would benefit from better metrics to evaluate the impact of investments. UW contributed another \$20 million. IHME's staff, now numbering nearly 100, built up a vast network of collaborators that included WHO: The new papers in *The Lancet* have 486 co-authors from 302 institutions.

The papers look at everything from DALYs to risk factors, causes of death, illness, and impairment, and how to weight the severity of nonfatal illnesses; their tables, maps, bar graphs, and charts reveal a multitude of intriguing patterns. Although mortality in children under age 5 has plummeted between 1990 and 2010, for example, more people now suffer from mental disorders and back pain. HIV/AIDS jumped from the 35th leading cause of death in 1990 to the sixth in 2010. Noninfectious diseases such as heart disease account for increasing amounts of "health loss." Several infectious diseases, including diarrhea and malaria, are on the decline.

Some of the findings are perplexing. Tuberculosis mortality, for example, has dropped steeply, but new cases have not. In 2010, road injury accounted for 10.7%

of deaths in males in the reproductive age bracket, but only 0.5% in females. Lower back pain ranks immediately below HIV/AIDS in DALYs.

Geographic differences jump out as well. Mortality in people of reproductive age changed little in Russia between 1970 and 2010, but skyrocketed in southern African (because of HIV/AIDS) and dropped in upper-income countries. Self-inflicted harm, including suicide, ranks

as the 13th most common cause of life-years lost worldwide but is rare in sub-Saharan Africa. Alcohol disorders have had a devastating impact in the former Soviet Union and parts of Latin America, where people drink more and liquor tends to be of lower quality.

Epidemiologist Peter Piot, who runs the London School of Hygiene & Tropical Medicine (LSHTM), says the absolute figures interest him less than the changes over time. "I don't care—and I don't think many people care other than disease advocates—whether 1.5 or 1.6 million die from a disease," says Piot, who serves on IHME's board. "What's important is what direction the world is going in and what's happening in my region."

Murray says that, after the fight over malaria, he doesn't anticipate much debate about other high-profile diseases, such as tuberculosis and HIV/AIDS. "The smaller diseases, those communities get more riled up," he says. "If our numbers are smaller, it's

THE WORLD'S TOP 12 HEALTH PROBLEMS

Ranked by Disability-Adjusted Life Years (DALYs)

Rank	1990	2010
1	Lower respiratory infection	Ischemic heart disease
2	Diarrhea	Lower respiratory infection
3	Preterm birth	Stroke
4	Ischemic heart disease	Diarrhea
5	Stroke	HIV
6	COPD	Low back pain
7	Malaria	Malaria
8	Tuberculosis	COPD
9	Protein, energy malnutrition	Preterm birth
10	Neonatal encephalitis	Road injury
11	Low back pain	Major depressive disorders
12	Road injury	Neonatal encephalitis

■ Communicable, neonatal/maternal disease
■ Noncommunicable disease
■ Injury

THE TOP 12 RISK FACTORS

Factors Causing the Greatest "Loss of Health"

Rank	1990	2010
1	Low body weight	High blood pressure
2	Household air pollution	Smoking
3	Smoking	Alcohol
4	High blood pressure	Household air pollution
5	Lack of breastfeeding	Low fruit consumption
6	Alcohol	High body mass index
7	Ambient particulate matter	High fasting plasma glucose
8	Low fruit consumption	Low body weight
9	High fasting plasma glucose	Ambient particulate matter
10	High body mass index	Inactivity
11	Low iron intake	High salt intake
12	High salt intake	Low nut/seed consumption

Rank and rile. GBD 2010 documents major shifts in DALYs and risk factors since 1990, but some doubt the new data.

going to hurt their bid for funding, so they get very restive. You'll have a million of those types of conversations."

They're already beginning. Peter Hotez, a pediatrician at the Baylor College of Medicine in Houston, Texas, who specializes in neglected tropical diseases, is a co-author on the GBD 2010 paper about DALYs. But he thinks the paper's estimates for schistosomiasis and Chagas—which he cares greatly about—are too low. Jamison says that IHME didn't properly factor in stillbirth in its calculations of under-5 mortality—"a conceptual hole of some magnitude."

Sandy Cairncross, a public health engineer at LSHTM who specializes in water and sanitation and who served on one of many expert groups for GBD, says that unsafe water and poor sanitation should have ranked much higher in risk factors. His concerns are so serious that he co-authored a commentary in this week's issue of *The Lancet* questioning whether policymakers

should even use GBD 2010's rankings of risk in their decisions.

Cairncross says that IHME dismissed much of the literature he selected that showed the important health benefit of delivering water to houses through pipes. "They only accepted one study in the world that got over their bar of scientific rigor," Cairncross says. "And that particular study apparently showed no significant effect on house connections, unlike most others that showed [disease] reductions of about 50%."

Black box step

Cairncross and several other critics say a fundamental problem with IHME's conclusions is that researchers used complex statis-

debtable issues—that has also frayed its ties with WHO. Initially, WHO envisioned working with IHME in a tight collaboration and even adopting its estimates. "We stepped into it because we thought it was a joint exercise," says Ties Boerma, director of WHO's health statistics and informatics, "but it became more of an IHME exercise."

A "briefing note" written by a WHO assistant director general last winter told WHO staffers that it would "not be appropriate" to be co-authors to the GBD 2010 papers or for WHO's logo to appear on IHME publications. According to the memo, obtained by *Science*, WHO developed serious concerns about the numbers in GBD 2010 after IHME researchers presented them to WHO

ceived as not being transparent." The report said that "IHME is viewed as a competitor vs. collaborator by many researchers in the health metrics field." Murray has gone so far as to suggest that WHO get out of the business of assessing GBD. "Bureaucracies don't do statistical innovation. Researchers do." But Boerma says that WHO will continue putting together its own GBD.

In a commentary in *The Lancet* package, Murray, Lopez, and other key IHME staff members say it's "reasonable and to be expected" that some contributors in an enterprise this large would disagree and choose not to be co-authors. But Murray challenges the accusation that IHME has not shared data and methodology. "The core

tenet throughout this collaboration has been that an open and voluntary process would provide for rigorous debate to ensure the best possible results," he says.

Hotez of Baylor says he has "a lot of sympathy" for Murray and his team. "It's incredibly complicated to bring all those investigators together," he says. And in the end, policymakers should keep the findings in perspective, Hotez adds. "It's one of several metrics that should be used when trying to control disease and exploring policy,"

he says, noting that it doesn't factor in economic costs of diseases, existing tools to combat them, or health system capabilities.

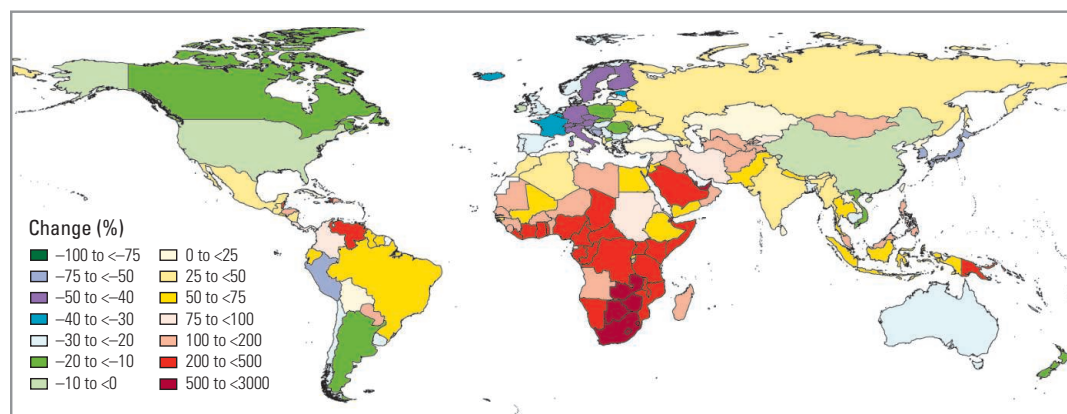
No fudged consensus

IHME intends to release another ocean of data in January, when it will report even more granular analyses of country-by-country information. It will also make a new interactive database publicly available that Murray says will lead people to explore questions that his team never imagined.

As debates about those data inevitably kick in, WHO plans to hold a meeting in February that will gather IHME scientists with experts from WHO and elsewhere to discuss how GBD 2010 reached its conclusions and how it differs from other estimates. Feachem says those discussions are exactly what's needed. "The last thing we want is fudged consensus," he says. "Some of these disagreements are healthy because they force tough questions. And that's how science works. In time we'll find a better outcome."

—JON COHEN

With reporting by Gretchen Vogel.



Diverse world. An analysis of changes in mortality among people of reproductive age over 3 decades reveals profound differences between countries.

tical models and computer analyses—that he calls a "black box step"—that baffle outsiders. The GBD 2010 paper on years lived with disability gives a flavor: "To address these challenges, we have developed a Bayesian meta-regression method, DisMod-MR, which estimates a generalized negative binomial model for all epidemiological data."

UCSF's Feachem says this "analytical sophistication" presents real challenges, but he contends that it's required because the jigsaw puzzle is so complicated. "By the nature of the beast, it will be very hard to get it to the point where the average epidemiologist with the average mathematical skills will be able to seriously reanalyze and arrive at different conclusions," he says.

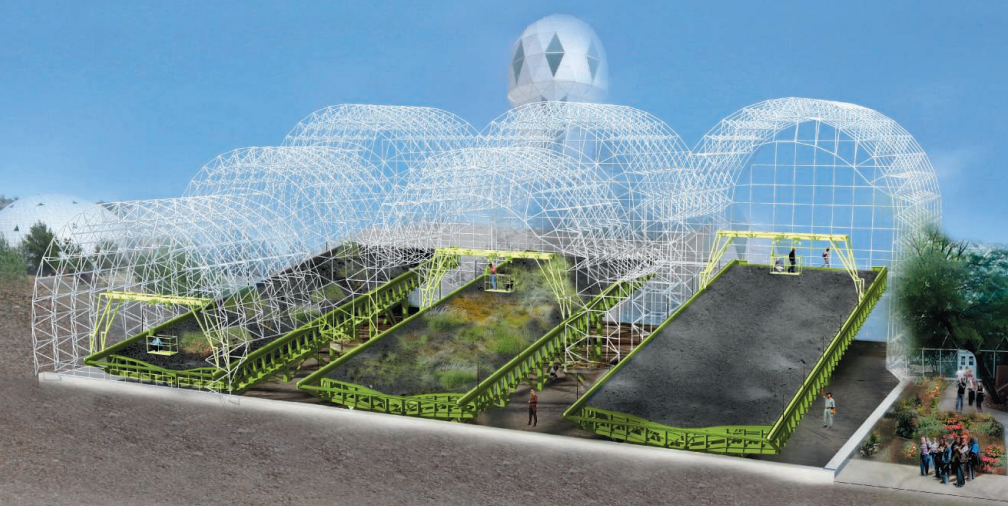
UW's Jamison says his former employer would mollify many critics if it embraced the transparency it espouses. "There's a lack of access to data," Jamison insists. "Their results can't be honestly checked and we don't have a capacity to interpret the underlying numbers."

The complaint is part of a bigger gripe about IHME's headstrong ways—and what some assert is Murray's over certainty about

staff members in September 2011. Based on those data, the memo says, big discrepancies between the GBD 2010 papers and WHO estimates were to be expected not just for malaria but also for child and maternal mortality, deaths due to neglected tropical diseases, vaccine-preventable diseases (including measles), cancers, and tobacco.

IHME subsequently adjusted its deaths for HIV/AIDS, Boerma notes, but in a commentary in this week's issue of *The Lancet*, WHO Director-General Margaret Chan says GBD 2010's estimates still "differ substantially from analyses by WHO and other UN entities." Boerma notes that one major discrepancy is that GBD 2010 estimates the total number of deaths annually at 52 million, WHO at 56 million. More differences may come to light as the published reports receive closer scrutiny.

IHME alienated several other erstwhile contributors along the way, and an external evaluation completed in November concluded that the institute "is not consistent in when and to whom it shares methods, data sources, [and] authorship and this is per-



RESEARCH FACILITIES

Experimental Landscapes Raise Stakes at Biosphere 2

The University of Arizona is betting that a unique trio of artificial hillsides will put an iconic greenhouse back on the map

ORACLE, ARIZONA—Perched high on a ladder, Peter Troch steps onto a girder and surveys a barren slope of black sand inside the new Landscape Evolution Observatory (LEO). “It’s hard to imagine that this will be a blooming desert one day,” says Troch, a hydrologist with the University of Arizona (UA) in Tucson. The artificial hillside, descending 5 meters over the length of a 30-meter-long steel frame, is chock-full of sensors and will let researchers explore fundamental questions about how water and soil—and eventually plants—interact under changing patterns of climate.

The broader hope is that the \$7 million observatory will reinvigorate research at Biosphere 2, which was erected more than 20 years ago as a controversial test-bed for human colonization of space. LEO now takes up one section of the 1.3-hectare complex of greenhouses and began operating last month. Just like Biosphere 2, the gleaming observatory is unprecedented in scale and boasts impressive engineering. “I think it’s very exciting,” says biogeochemist Susan Brantley of Pennsylvania State University, University Park, who is not involved. “I also think it’s kind of audacious.”

Funded entirely by a private donation, LEO is a gamble. No one knows exactly what will happen as the decade-long experiment proceeds or how long its sensors will last. It’s also not clear whether the facility will attract broader interdisciplinary collaborations or the dedicated funding needed to reach its full potential. But Henry Pollack, a geophysicist at the University of Michigan, Ann Arbor,

and chair of Biosphere 2’s advisory board, is optimistic. “This is a moment of opportunity for Biosphere,” he says. “A big program is off and running.”

Biosphere 2 is no stranger to uncertainty and change. The last team of Biospherians emerged from the sealed facility in 1994 after 6 months in isolation, and the building became a tourist attraction. Columbia University tried for several years to run a financially viable research campus, but it ended up mothballed. When the University of Arizona took over in 2007, administrators wanted to create a unique infrastructure for research. A scientific advisory committee conceived the idea for a trio of artificial hillslopes (*Science*, 8 July 2011, p. 146).

Researchers studying watersheds typically have had imperfect options. Lab experiments are small-scale, and conditions

S Video about LEO by author Erik Stokstad.
www.scim.ag/6113_vid

at field sites cannot be fully controlled. Housed inside three cavernous, glass-enclosed bays, the hillslopes are more realistic in size yet still offer the ability to manipulate the environment. The resulting observations will help researchers study questions such as how the flow of underground water is affected by plant growth and by chemical changes in the soil. In addition, scientists will test computer models of watershed hydrology and vegetation dynamics, important for predicting environmental change from global warming, Troch says.

Building LEO took careful planning. The

Coming to life. Three densely instrumented hillsides will reveal connections between soil, water, and plants.

team selected a volcanic basalt from northern Arizona that is likely to weather quickly—a crucial factor when hoping to observe soil development in just a decade—and had it ground to sand. After much debate, the staff decided to build three identical hillslopes. This approach provides greater statistical rigor, but limits the types of experiments that are possible. Thanks to the design of Biosphere 2, air temperature and humidity will be independently controlled for each hillslope, allowing the simulation of various desert climates. Overhead sprinklers can mimic various patterns and intensities of rain.

Researchers will have a close view of what happens as the water flows through the hillslopes, which each contain 1855 sensors and sampling devices for studying water, gas, and soil. “This is really powerful for understanding what’s going on in the subsurface,” says LEO lead scientist Stephen DeLong. Using these sensors, the team can map the flow of water. And because the steel frame of each hillslope rests on heavy-duty scales, researchers can measure its weight to help monitor how much water is evaporating.

The big question for LEO is its future funding. At the moment, the annual research budget is expected to be about \$500,000—enough to get going, but less than Troch’s ideal annual budget of \$3 million. The money comes from billionaire oilman Edward Bass, who funded the \$200 million construction of Biosphere 2 and also picked up the tab for building LEO. Enough of his gift remains to operate LEO for about 5 years, says Joaquin Ruiz, the dean of UA’s College of Science and director of Biosphere 2. “What is a real problem as we go forward is how to sustain a healthy research enterprise.”

Ruiz says that LEO could become a facility for a wide range of scientists, which might help it attract long-term support from the National Science Foundation (NSF), for example. Yet, that vision represents a “very serious challenge,” according to Teofilo “Jun” Abrajano of NSF’s Directorate for Geosciences. The reason is it may not be easy to add other types of experiments without disturbing what’s already going on, Abrajano explained here last month at a panel discussion after LEO’s opening ceremony. Troch, who is Biosphere 2’s science director, says he plans to reach out for more input on experimental design. Ultimately, he hopes, a diverse community of research will spring up as LEO’s hillslopes evolve.

—ERIK STOKSTAD

LETTERS

edited by Jennifer Sills

Cloudy Forecast for Weather Satellite Data

IN THE NEWS FOCUS STORY “WEATHER forecasts slowly clearing up” (9 November, p. 734), R. Kerr nicely summarizes how the growing improvement in prediction is coming from a focus on “more computer power, the assimilation of radar observations, and more physically realistic models.” He emphasizes that better assimilation of satellite data is a key element of improved forecasting.

Unfortunately, these potential improvements will have little effect on forecasts if the basic data set from the existing polar-orbiting weather satellite system is not available. In a report issued in June 2012 (1), the U.S. Government Accountability Office noted that “data from this system is the predominant input to numerical weather prediction models” and warned that “there will likely be a gap in satellite data lasting 17 to 53 months” when the National Polar-orbiting Operational Environmental Satellite System (NPOESS) Preparatory Project satellite ceases operations and NOAA’s new satellite system (the Joint Polar Satellite System) launches. The report also notes that there are “potential satellite data gaps in DOD [Department of Defense] and European polar satellite programs which provide supplementary information to NOAA forecasts.” These gaps are a grave problem and would seriously degrade weather forecasts. Therefore, the agencies responsible for weather forecasting—NOAA, DOD, and NASA—should make filling the polar satellite data gaps the first priority in order to ensure that future forecasts are as good as possible.

D. JAMES BAKER

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Big Brains, Little Bodies

IN THE NEWS FOCUS STORY “WHY ARE OUR brains so big?” (5 October, p. 33), M. Balter claims that “the size of the *Homo sapiens* brain outstrips that of any other animal” once an adjustment is made for body weight. When expressed as a percentage of body mass, the brain masses of some small mammals considerably exceed the approximate 2% value for humans [e.g., the brains of Eurasian harvest mice (*Micromys minutus*) comprise

roughly 10% of their total mass] (1, 2). Many invertebrate animals have brains that are relatively even larger (3), including tiny ants with brains that account for nearly 15% of their body mass (4). Perhaps this is one reason Darwin noted that “the brain of an ant is one of the most marvelous atoms of matter in the world, perhaps more marvelous than the brain of man” (5).

WILLIAM T. WCISLO

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Response

WCISLO IS BASICALLY CORRECT IN REGARDS TO the actual ratios of brain and body weight. What I meant to refer to was the fact that the relationship between brain size and body weight is not a linear one, an idea expressed in the Encephalization Quotient (EQ) referred to in the story. Humans have a much higher EQ than any other animal; the EQ of a mouse is actually very low.

MICHAEL BALTER

Pushing the Planetary Boundaries

IN HIS PERSPECTIVE “A MEASURABLE PLANETARY boundary for the biosphere” (21 September, p. 1458), S. W. Running proposes a new “planetary boundary” definition for land use based on net primary (plant) production (NPP). On the face of it, NPP is a much more robust scientific indicator of biospheric constraints to human populations than the arbitrary 15% of global ice-free land converted to crops proposed by Rockström *et al.* (1). Earth’s NPP is fairly constrained as a global process and is a critical resource for both human and ecological systems. Humans need biomass as food, feed, fiber, and bioenergy. In ecosystems, biomass is the ultimate energy resource sustaining the metabolism of all species on Earth and replenishing the carbon stored in biota and soils (2).

However, basing policy-relevant boundaries for land use on NPP is not straightforward. First, NPP is a poor indicator of food



NPOESS Preparatory Project satellite (artist's rendition).

and other resources available for consumption by humans. For example, the NPP of a tropical forest is exceedingly high, yet the food resources for humans are far less in a tropical forest than if the land were converted to a field of soybeans with lower NPP. Second, NPP can be both reduced and increased by human activities in agricultural and other land-use systems, and it is more dynamic at regional scales and over longer time spans than indicated by short-term global averages (3–5). Third, improvements in agricultural and processing technology as well as livestock management allow major increases in the efficiency with which NPP is converted into the raw materials that humans use (6).

All of this suggests that NPP—despite being a critical, ultimately limiting resource, as argued by Running—is a moving target rather than a fixed boundary imposed by natural laws. It is the access to technology, land, and capital to increase food production that is determining the amount of NPP usable by humans rather than any absolute biophysical boundary to NPP overall. Thus, sound indicators are required that put these complex links between human activities and NPP into focus.

Furthermore, there is no one-to-one correspondence between human appropriation of NPP and sustainability of land use: Intermediate, perhaps even high, levels of human appropriation might be sustainable, and degradation can occur even at low levels of human appropriation if land management is poor. Using NPP to indicate boundaries might therefore result in silent degradation remaining undetected (7) and a disincentive for the development of innovative, sustainable but intensive agricultural systems. By ignoring the well-known potentials for more efficiently using these resources (8, 9), Running all too easily argues that further intensification is not possible due to constraints entailed by boundaries for water, nitrogen, and phosphorous.

Using NPP as an integrating boundary, as proposed by Running, conceals the range of available options for land use. NPP-based metrics are complements, not substitutes for indicators of boundaries such as N pollution, climate change, soil degradation, or biodiversity loss. The trade-offs between them require monitoring all boundaries

separately to avoid displacement and leakage effects, and fixing one problem by creating others. Although the prospects of a global and comprehensive measure of human limits based on NPP is appealing, it is not sufficient to grasp the complex, dynamic nature of human interactions with the Earth system and the trade-offs humanity must make on its road to a sustainable future.

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Response

ERB *ET AL.* DO NOT CHALLENGE MY ASSERTION that global NPP may be a constant boundary. Rather, they argue that partitioning the 38% of global NPP appropriated for human use will be complex and that the efficiency of agricultural crop production is variable.

The fraction of agricultural NPP consumable by humans as food varies widely by crop type, as Erb *et al.* have previously reported (1, 2). In addition, more efficient use of current irrigation and fertilizer will allow agricultural food output to be improved in some regions. However, there is much evidence cited in the original Rockström analysis (3) that nitrogen and phosphorus cycles may already be saturated, so increasing the use of fertilizer to enhance future NPP to satisfy growing demand may be environmentally

counterproductive. Many studies also warn that major new sources of irrigation water are not likely and that dewatered rivers and groundwater depletion suggest that even current levels of global irrigation are not sustainable (4–6). Thus, whereas the current NPP for human use may be a “moving target” for the reasons Erb *et al.* suggest, the total global NPP does seem like a planetary boundary that, once reached, humans cannot extend.

There appears to be adequate capacity for global food production as human populations and living standards increase over the next century, assuming increasing efficiency and reducing waste, and (if needed) potentially devoting the remaining 10% of available NPP to agriculture (7, 8). However, if this same 10% of available global NPP were all devoted to bioenergy (not considered by Erb *et al.*), it would not even satisfy current global energy consumption, regardless of what type of conversions and final fuels were produced (9). A future of food competing against bioenergy for the remainder of available global NPP seems likely.

Planetary boundaries have been criticized as being conceptually attractive but lacking in measurable global metrics. Global terrestrial NPP provides a measurable and policy-relevant boundary, a real “limits to growth,” which strategic economic thinking can no longer ignore (10).

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Letters to the Editor

Letters (~300 words) discuss material published in *Science* in the past 3 months or matters of general interest. Letters are not acknowledged upon receipt. Whether published in full or in part, Letters are subject to editing for clarity and space. Letters submitted, published, or posted elsewhere, in print or online, will be disqualified. To submit a Letter, go to www.submit2science.org.

CORRECTIONS AND CLARIFICATIONS

Reports: “A large-scale model of the functioning brain” by C. Eliasmith *et al.* (30 November, p. 1202). The name of the sixth author was incorrect. It should be Yichuan Tang. The HTML and PDF versions online have been corrected.

News Focus: “Making sense of a senseless act,” by M. Hvistendahl (23 November, p. 1025). Suicide researcher Matthew Miller is quoted as saying that mental illness may be underdiagnosed in Asia for reasons that aren’t fully understood. He had said that there may be differences in the recognition, reporting, and incidence of mental illness in Asia, not that mental illness is underdiagnosed. The HTML and PDF versions online have been corrected.

COMPLEX SYSTEMS

Boldly Going Beyond Mathematics

Christoph Adami

Before the computer age, progress in science was achieved mainly by the interaction of two processes: gathering empirical data and crafting theoretical descriptions of the forces and agents that give rise to our observations. The story of how theories—perhaps inspired by new observations—make falsifiable predictions that are then compared to further observations and, possibly, refinements of the theory has been told many times. This theory-observation-refinement cycle has arguably provided many of our most profound insights into how the universe works, in particular in physics and chemistry. It has not worked so well for developing our understanding of complex systems.

For one thing, complex systems do not easily lend themselves to analysis, the process of taking apart a system and examining its components individually. If taken apart, many complex systems lose precisely the character that makes them complex. The essence of these systems, then, seems to lie not in the nature of their components but in how the components interact—across different hierarchies, in synergistic and antagonistic manners. The agents within these systems are heterogeneous (think participants in a market economy or molecules within a cell), and their behavior is influenced by the type and quantity of other agents nearby. Such systems defy description with the traditional tool of theory builders: mathematics. Instead, they must be modeled by taking into account the rules of interaction, the natures of the agents, and the way the

agents, rules, and ultimately whole systems came about. In his *Signals and Boundaries: Building Blocks for Complex Adaptive Systems*, John Holland proposes that computational modeling is the appropriate tool not only for describing but, fundamentally, for understanding such systems. In particular, he argues that this modeling approach is in no way inferior to a mathematical one. Rather, he advocates that the computational modeling of signal-boundary systems (which I will describe in more detail below) goes where mathematics cannot go while being no less rigorous, no less exact.

Now 83, Holland, a computer scientist and psychologist at the University of Michigan, has thought about complex systems for a long time. One of the fathers of the field of evolutionary computation, he is widely credited for having invented a machine-learning method based on the genetic pro-

cesses of inheritance, mutation, recombination, and selection: the genetic algorithm. He has also written extensively about cognitive science, the brain as a complex system, and adaptation in general (1). Indeed, perhaps the most outstanding aspect of the computational approach that he describes is that it must be evolutionary: the signals and the boundaries that are the system must be the result of a coevolutionary process.

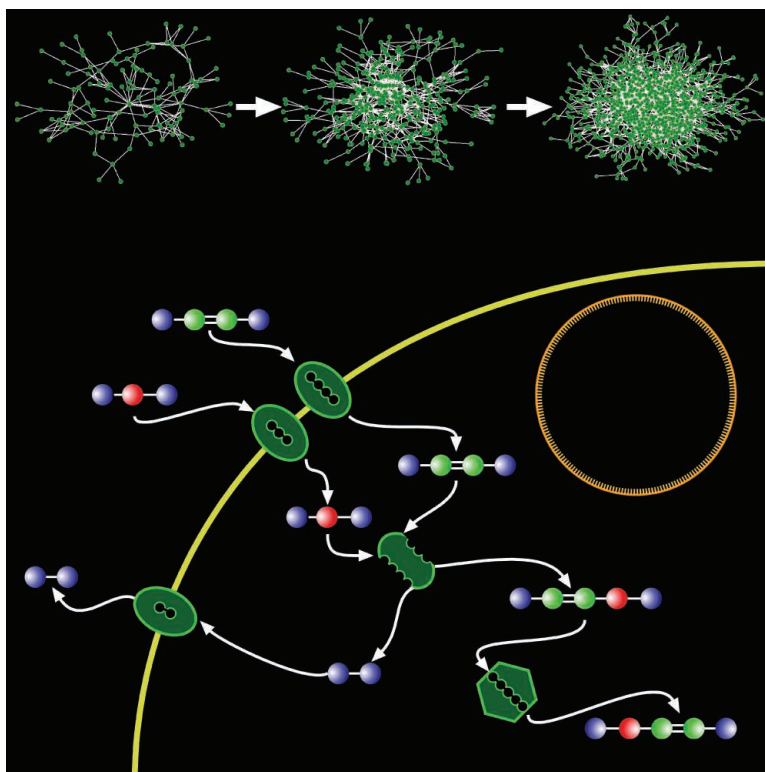
But let me step back and describe what Holland means when discussing signal-boundary systems. In any complex system, agents make decisions that affect not only their own fate but also the actions and decisions of other agents—and the very system within which these decisions are made. The reach of these actions is not infinite, and the source of the information that was used to generate them cannot be infinitely remote. The dynamics of the system are influenced by both the signals and the boundaries within which the signals originate or are received. In a cell, for example, there are myriad agents (proteins) that process signals (small molecules and other proteins), and they interact with cellular or organelle boundaries. In a society, the agents are a diverse set of people with different roles, and the signals through which they interact depend on (and are shaped by) the boundaries (of home, city, or country). Naturally, boundaries are not impermeable, nor are they permanent. The book focuses on understanding how these boundaries transmit signals and how boundaries within boundaries give rise to hierarchically organized systems.

Because the formalism for the description of signal-boundary systems should, in Holland's view, be applicable to any complex system (whether a cell, an organism, a company, or a country), it is necessarily abstract. Indeed, it is an amalgamation of a number of toolboxes that have been developed over time, including classifier systems, genetic algorithms, urn models, and several others. Holland calls such amalgams "dynamic generated systems," because the ultimate rules agents use to process signals are dynamically

Signals and Boundaries Building Blocks for Complex Adaptive Systems

by John H. Holland

MIT Press, Cambridge, MA,
2012. 316 pp. \$40, £27.95.
ISBN 9780262017831.



An example. Signals and boundaries in the artificial cell model (3) and the metabolic networks that evolve in them.

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generated within the system. They are not designed. Nor are the boundaries designed: they should emerge in the coevolutionary process that selects for agents that make appropriate decisions given the environment within which they operate.

This may sound like a radical approach to modeling, in particular because Holland emphasizes that these models do not, strictly speaking, model anything. Rather, they are exploratory in nature: They are aimed at exploring the consequences of the underlying model assumptions. If the predictions (after evolving the model through thousands if not millions of generations) contradict our observations, we have to go back and question the model assumptions. In this way, computational modeling plays exactly the role that theory has played, only packed with the power of computers that mathematics alone cannot achieve.

But the approach is less radical than one might think. Such approaches have been quite successful in the past. For example, the Avida system that was designed to study basic evolutionary dynamics (2) can be seen broadly as an instantiation of such a signal-boundary system, complete with a symbolic basis, grammar, niches, ecologies, signal processing, and, of course, evolution. And Avida is exploratory in the sense that it has generated hypotheses about the natural world that subsequently have been tested there. Another candidate signal-boundary system is the artificial cell model (3). In it, nearly every one of Holland's requirements for such a system has been implemented, and it too can be used to make falsifiable predictions about the natural world even though the model itself is artificial, an abstraction.

Signals and Boundaries hearkens back to another influential book within the field of complexity research, Herbert Simon's *The Sciences of the Artificial* (4), which in its third (1996) edition received a new chapter dealing with the genetic algorithms and adaptive systems championed by Holland. But Holland's book is different in an important aspect. It becomes technical when mathematics is needed, and Holland does not shy away from introducing complicated computational structures. In these passages (and chapters), it becomes less of a science book for the interested lay person (as Simon's book is) and more of a textbook. In this respect it bears some resemblance to Douglas Hofstadter's *Gödel, Escher, Bach* (5), which (in the field of cognitive science) also did not shy away from being both technical and ambitious. However, Holland's book is much more succinct, clocking in at 301 duo-

decimo pages, compared with the 777 quarto pages of Hofstadter's classic.

Signals and Boundaries is indeed an ambitious book because Holland advocates (correctly in my mind) a culture that does not treat computational approaches as "mere models," as if they were inferior or less rigorous than purely mathematical treatments. Perhaps it would have been strengthened had the author drawn more from existing modeling endeavors that have followed his vision almost to the t. But it is still a "remarkable achievement" (as Holland's colleague Robert Axelrod calls it on the dust jacket)—not the least because it would qualify as such had it been written by someone much younger.

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COMPUTER SCIENCE

Casting a Wider Net

Lynn Andrea Stein

Mung Chiang's *Networked Life: 20 Questions and Answers* has an intriguing premise and an ambitious vision. As the book's title suggests, Chiang sets out to explain the various technologies that underpin all facets of our interconnected society. His approach begins with the questions of the subtitle—20 curiosities of how (and why) it is that our networked world works. He goes on to provide multilayered answers intended in turn for the interested public, for undergraduates, and for a sophisticated advanced readership. To further up the ante, the book is also the text associated with a massive open online course (MOOC)—Networks: Friends, Money, and Bytes (1)—offered for free

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through Coursera (with videos also on YouTube) alongside its more conventional incarnation at Princeton University.

Chiang intends for the book to provide something for everyone. Its topic matter ranges from Netflix's movie recommendations to Verizon's pricing model, from building a Twitter following to Skyping for free. *Networked Life* explores phenomena that may seem obscure to the general public—such as cloud computing, TCP/IP (Transmission Control Protocol/Internet Protocol), and CDMA (Code-Division Multiple Access) cell phone infrastructure—as well as pop culture memes like "going viral" and the old chestnut about six degrees of separation. The topics are broad ranging and the influences equally eclectic. Each of the 20 questions is meant to draw in readers by addressing some familiar phenomenon, pulling aside the curtains to show how the technology really works.

The book has its roots in an innovative course taught by Chiang to undergraduate electrical and computer engineers at Princeton University. Bridging disciplinary boundaries, introducing mathematics only as needed, and drawing connections across disparate phenomena using graph, optimization, game, and learning theory, this unusual course teaches in an integrated and application-specific way. Chiang's framing of the material as 20 intriguing questions about networks, their architectures, and associated phenomena ties theory to practical systems that students encounter every day. The shift from comprehensive content coverage to thematically organized material with real-world applications is a step toward the future of learning. Just in time trumps just in case in a world where knowledge is increasingly available, where which and why and how become more critical than what. Chiang's course

surely pushes the boundaries of the traditional lecture, and the book similarly is meant to be a next-generation work.

Chiang would like it to serve as both an undergraduate textbook and a broadly accessible popular work. The opening part of each chapter offers "a short answer"

devoid of mathematics, up to nine pages, meant to give the novice a sense of how the rest of the explanation will proceed. Chiang expands on this in "a longer answer" (sometimes occupying fewer pages) suitable for "primarily juniors and seniors in electrical engineering and computer science" or others with similar background including "differen-

Networked Life
20 Questions and Answers
by Mung Chiang
Cambridge University Press,
Cambridge, 2012. 504 pp. \$45,
£27.99. ISBN 9781107024946.

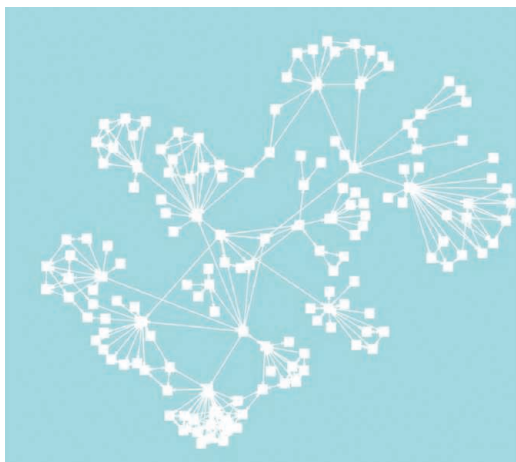
tiation and linear algebra.” A section of worked examples—applications of mathematical theory to particular contrived situations that Chiang suggests should be familiar to any student in mathematics, science, or engineering—is followed by advanced (graduate-level) material. One finds a very helpful, short summary hidden between the end of this advanced material and the chapter’s selected references, which are themselves followed by exercises.

As a textbook, the work is appealingly narrative—certainly an easier read than traditional approaches—but it consequently lacks the cues that would make it a useful reference.

Principles are not separated or boxed for revisiting, and the presentation complicates attempts to locate particular points or to identify antecedents when one becomes confused (as, e.g., by the differences intended by the matrices $\mathbf{R}$, $\hat{\mathbf{R}}$, and $\tilde{\mathbf{R}}$). Importantly, the text is almost identical to the video lectures, making the two media reasonable alternatives to one another. It is harder to imagine how this text would serve for a different course.

As a popular work for a nonmathematical audience, *Networked Life* is strikingly brief (2), perhaps slighted by what is left to the more technical sections. Sometimes, the text of the short answers for the “casually curious” is effective, as when (in “How can I pay less for each GB?”) Chiang explains time-dependent pricing, analogous to old-fashioned telephone night-time calling rates, and when (in “How can Skype and BitTorrent be free?”) he describes how peer-to-peer networks incur reduced costs (relative to commercial networks) by leveraging participant nodes to provide service. At other times, these “easily understandable” sections are merely tantalizing, pointing in the direction where answers lie while failing to provide any real intuition to the nonmathematical audience. Occasionally, the nominally accessible portion of the text is in fact obscure or, assurances aside, relies on mathematical understanding. Many of these sections run only a few pages; the shortest (for “Does the Internet have an Achilles’ heel?”) is three words: “It does not.” The description of the network stack (in “How does traffic get through the Internet?”) is among the most fascinating of the short answers Chiang provides. It is also, perhaps not coincidentally, among the longest.

Networked Life is very much a snapshot of an instant in time, from its topic (timely and trendy at this very moment) to its attempt to defy conventional categorization (is it a pop-



César Hidalgo's *SINGLEBLUE* (2006/2007).

ular work or a textbook?) to its embedding in an early-stage MOOC. But education is changing, textbooks are in transition, and networks and the technologies discussed in the book are evolving. It will not be long before a new generation of technologies makes us wonder at everything about this particular pile of paper between hard covers. As much as Chiang intends the book to be a model for the future, it may take many of us back to the anonymous lecture halls of our college experiences. Viewing the online videos (through Coursera or YouTube) will further solidify such memories—they feature scrawled handwriting over minimalist slides while the lecturer’s voice fills the background. The online course is an odd throwback for such a forward-looking text. The experience seems less dynamic than sitting in the lecture hall in person, albeit offering the convenience of being viewable around the world and in one’s pajamas. What is tantalizing here is not the current implementation but the promise of what is to follow.

MOOCs are reputed to be changing education. Availability to anyone, anywhere, at any time is certainly a huge step—and one enabled by those very technologies at the core of *Networked Life*. But more is possible. Coursera’s founders describe their intention to leverage this massive audience, to explore artificial intelligence-enhanced, crowdsourced, peer assessment (including more typically subjective material, such as essays) and to use the enormous cache of click-by-click data to ultimately customize learning (3). Rival MOOC Udacity (4), on the other hand, emphasizes course content and activities that are more explicitly tailored to the online environment, incorporating interactive exercises and moving away from pure lecture pedagogy (5). In the end, online education will likely include all of these aspects as well

as additional, still unforeseen innovations. As education changes, so too will pedagogies.

Yet, as ambitious as Chiang has been in conceiving his content, his present project remains tied to the one-size-fits-all approach of broadcast, and its transfer to paper and to MOOC infrastructure has so far done little to remedy this. The first steps are elsewhere. Chiang’s own Princeton course now uses a flipped format, with students solving problems in the classroom and using YouTube and Coursera for out-of-class first presentation of topics. Instructors presumably act as coaches, customizing experiences for individual students. Open-ended problems; student self-direction; and projects providing application, design, and creative implementation opportunities—such as those contemplated by Coursera and Udacity for their futures—would take this material still further. Online, there are myriad opportunities for future technologies to bring mass customization, data mining for differentiated instruction, peer-to-peer collaboration, or automated interactive infrastructure for active student practice. On campus—with physical facilities for experimentation and exploration, content presentation accessible anytime online, and the benefits of communal collaboration—the possibilities for new, individualized, face-to-face educational experiences are endless.

A friend relates that half the world enjoys a steaming hot cup of tea, while the other half appreciates their tea in an icy cold glass. But no one, my friend suggests, really likes lukewarm tea. Attempts to serve too many audiences, however well conceived, can ultimately compromise one’s ability to genuinely satisfy anyone. I am extremely glad that *Networked Life* exists, just as I am enthusiastic about the emergence of the MOOC. I appreciate these things not so much for what they are—here, now—as for the path that they highlight for us and for the future incarnations of technology and learning to which they lead. As it happens, I do drink a lot of lukewarm tea, a hazard of absent-minded professorship. That does not prevent me from wistfully imagining how much hotter (or cooler) *Networked Life*’s next generation will be.

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10.1126/science.1230710

CLIMATE CHANGE

The Greening of Insurance

Evan Mills

Every sector of the economy telegraphs climate risks to its insurers. In turn, climate change stands as a stress test for insurance, the world's largest industry, with U.S. \$4.6 trillion in revenues, 7% of the global economy (1–6). Insurers publicly voiced concern about human-induced climate change four decades ago (1). I describe industry trends, activities, and promising avenues for future effort, from a synthesis of industry progress in managing climate change risk [see supplementary materials (SM)].

Increasingly, multifaceted weather- and climate-related insurance losses involve property damage, business disruptions, health impacts, and legal claims against polluters. Worldwide, insured claims that were paid for weather catastrophes average \$50 billion/year (about 40% of total direct insured and uninsured costs); they have more than doubled each decade since the 1980s, adjusted for inflation (7, 8). Insurers must also adjust to risks emerging from society's responses to climate change, including how structures are built and energy is produced.

Where there are risks, there are also opportunities. Responding to the push of shareholders and regulators and the pull of markets, a trio of global initiatives [United Nations Environment Programme Finance Initiative (1995), ClimateWise (2007), and the Kyoto Statement (2009)] has aggregated 129 insurance firms from 29 countries (table S1). Member commitments include supporting climate research, developing climate-responsive products and services, raising awareness of climate change, reducing in-house emissions, quantifying and disclosing climate risks, incorporating climate change into investment decisions, and engaging in public policy. Since the mid-1990s (3), these and many other insurers, reinsurers, intermediaries, brokers, industry associations, catastrophe-loss modelers, and regulators have engaged in this work (see the figure) (fig. S1, A to C), often in partnership with universities, development agencies, nongovernmental organizations, foundations, think tanks, and governments (9). These increasingly sophisticated efforts were sustained through the economic malaise of the past few years; one-fifth of the activities identified in the figure began after 2008.

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Climate Science, Adaptation, and Mitigation

As past experience is an ineffective predictor of future losses, many insurers are using climate science to better quantify and diversify their exposure, more accurately price and communicate risk, and target adaptation and loss-prevention efforts (table S2). Insurers also analyze their extensive databases of historical weather- and climate-related losses, for both large- and small-scale events (7–11). Insurers from North America, Asia, and Europe have expanded their collaborations through the three latest Intergovernmental Panel on Climate Change assessments into projects such as harmonizing economics-based insurer catastrophe models with climate models. Insurers' models extrapolate historical data rather than simulate the climate system, and they require outputs at finer scales and shorter time frames than climate models.

Insurers can reactively adapt to rising losses by tightening availability, prices, and terms. Instead, some have sought to help vulnerable customers improve their resilience to a changing climate. Strategies include financial and physical risk management, often in collaboration with noninsurance entities (table S3). Insurers have championed a broadened definition of sustainability that includes resilience to disaster and a low carbon footprint. Beyond signaling that loss-prone development is unsustainable, insurers are supporting interventions with benefits for both emissions reduction and adaptation (table S4 and fig. S2). Integrated actuarial and environmental science is enhancing adaptive capacity to climate change in the developing world, where poor populations enjoy little access to insurance. Decades ago, public and nonprofit sectors offered microinsurance (small premiums for modest coverage), with commercial insurers later adding tens of millions of policies for life, health, and property (table S5). Some employ parametric and index-based triggers for climate-sensitive crops and livestock by using remote sensing. Others promote adaptation, e.g., improved soil management.

Numerous insurers aim to curb greenhouse-gas emissions from homes, businesses, transport, industry, and agriculture (table S5). They have brought to market at least 130 products and services for green buildings. Many pay claims that fund rebuilding to a

Insurance industry trends show how market-based mechanisms support climate change mitigation and adaptation.

higher level of energy efficiency after losses. Insurers have introduced at least 65 offerings for renewable energy systems.

Some climate-change mitigation technologies align with lower-risk behavior. Nearly 3 million pay-as-you-drive policyholders enjoy more accurate roadway accident premiums using telematics to verify distances driven. This price signal could reduce U.S. driving by 8%, worth \$50 to \$60 billion/year, thanks to reduced congestion and lower probability of accidents, while reducing cross-subsidies from those who drive less than average to those who drive more (12). Risk-based premium credits are also offered for low-emissions vehicles and green buildings (table S5).

Other products insure financial shortfalls if energy savings or low-emissions power generation projects underperform or manage risks in carbon-trading transactions, ranging from carbon release from wildfires to infrastructure appropriation by foreign governments. Insurance strategies assuming these risks and minimizing losses align with the broader policy objectives of verifiable, bankable, and persistent emissions reductions.

Technology, Governance, and Policy

When risks are too great or undefined, insurers withdraw coverage or increase prices. Climate change mitigation and adaptation present dual challenges in this regard: unintended risks (e.g., nuclear power and weapons proliferation) and climate vulnerabilities (e.g., biofuels and water needs) (tables S6 and S7). Insurers abhor unquantified and unpriced risks, as well as market distortions, such as equally subsidizing technologies that have divergent risk profiles (13).

Emerging technologies lack the operational history desired for underwriting. The most unwieldy of these are "climate-engineering" techniques, ranging from carbon capture and storage (CCS) to artificially modifying the radiative properties of the atmosphere. Insurers have entered the CCS market in a circumscribed manner, excluding riskier strategies or financial arrangements, limiting coverage to short time frames, and ceding long-tail risks to the public sector. Conversely, energy efficiency is arguably the lowest-risk mitigation strategy (followed by renewables), with abundant benefits (14). Societal dithering forces reliance on approaches that are riskier and less amenable to insurance underwriting.

Insurers are dually exposed to internal governance risks (e.g., underestimating climate-related losses) and those taken by their customers (e.g., polluters). More than one in four corporate directors anticipate liability claims stemming from climate change (15). Litigation often requires insurers to furnish legal defense and to pay damages. Insurers have responded with new liability products and by excluding climate-change claims where customer behaviors are unduly risky. Insurance regulators and investors are seeking climate-risk disclosure (table S8 and fig. S3), compelling insurers to formally consider climate change in operational, business, and investment practices. Liability risks are rising as climate science becomes more settled.

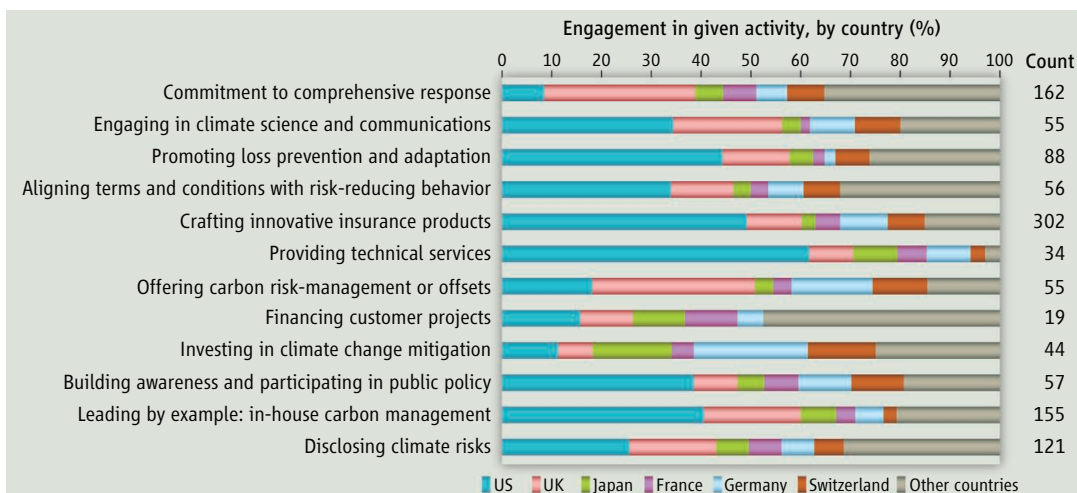
With \$25 trillion in assets—equal to global mutual funds or pension funds—insurers are central players in world financial markets. They have invested at least \$23 billion in emissions-reduction technologies, securities, and financing, plus \$5 billion environmentally focused funds (table S9 and fig. S4).

Emissions from insurers' energy-intensive buildings, data centers, and business travel are 12 megatons CO₂/year with a 10-fold variation in carbon intensity (per unit of revenue) across companies (9). Scores of insurers have reduced their emissions, with at least 26 carbon neutral (table S10 and fig S5).

Insurers have influenced public policy, striking agreements on pricing risk and government's role in risk management and shaping land-use planning and energy policy in many countries. They have engaged in climate policy forums since the mid-1990s (2, 3), including participation in the international climate negotiation process. Lloyds of London is one of the more prominent; they view climate change as the industry's number-one issue (5). Insurers are uneasy with mercurial policies on natural hazards and energy. Shifts in public incentives or indemnity practices can adversely influence risk-taking (moral hazard), heightening insurers' exposures.

From Risk to Opportunity

Climate-focused efforts have benefitted millions of insurance customers and have mobilized billions of investment dollars, although public and policy-maker engagement in these



Global insurance industry engagement in climate change adaptation and mitigation activities. As of late 2012, a total of 1148 initiatives have emerged (largely in the past decade) from 378 entities in 51 countries, representing \$2 trillion (44%) of industry revenues. Count represents number of activities in each category. Details in SM.

efforts remains low. Little climate-related innovation has occurred in certain market segments, e.g., life and health (microinsurance being an exception), maritime, aviation, and heavy industries. Greater scale is needed if the insurance industry is to realize its potential.

Independent auditors found an 88% compliance rate among signatories of the ClimateWise principles (table S1) (16). Yet, many companies remain on the sidelines or offer only token gestures, perhaps because of insufficient demand, ideological discomfort with policy responses, inadequate science literacy, or inertia to institutional change. Insurers face external barriers as well. Some regulators and consumer groups resist risk-based pricing and insurer innovations (17).

It is argued by some that private insurers have not effectively advanced climate change mitigation and adaptation and that the risks may even become uninsurable (18). Mandatory climate-risk disclosure identified a broad consensus on the relevance of climate change among U.S. insurers, but only one in eight companies have a formal strategy.

Public insurers could be similarly criticized (19). As insurer of last resort (e.g., \$1.3 trillion coverage for flood and \$115 billion for crops in the United States), they could learn from their private counterparts. Governments could boost demand for market-based "green insurance" by using it in their own operations.

Promising scientific frontiers include loss modeling under future climates, preferably on a public-domain platform, to yield better economic assessments and policy pathways. Lacking are comparative risk-assessments of climate-change response options to inform research and development and policy needs and to determine their insurability.

The insurance sector is a global clearing-house for climate risks that affect every underwriting area and investment. Where insurers recoil in the face of climate change, consumers will encounter acute affordability issues accompanied by huge holes in this societal safety net. But insurers' efforts to date demonstrate that market-based mechanisms can support greenhouse-gas emission reductions and adaptation to otherwise unavoidable impacts.

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Supplementary Materials

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HISTORY OF SCIENCE

Is Science Mostly Driven by Ideas or by Tools?

Freeman J. Dyson

Thomas Kuhn was a theoretical physicist before he became a historian. He saw the history of science through the eyes of a theorist. He gave us an accurate view of events in the world of ideas. His favorite word, “paradigm,” means a system of ideas that dominate the science of a particular place and time. A scientific revolution is a discontinuous shift from one paradigm to another. The shift happens suddenly because new ideas explode with a barrage of new insights and new questions that push old ideas into oblivion. I remember the joy of reading Kuhn’s book, *The Structure of Scientific Revolutions*, when it first appeared in 1962. It made sense of the relativity and quantum revolutions that had happened just before the theoretical physicists of my generation were born. Those were revolutions led by deep thinkers—Einstein and Heisenberg and Schrödinger and Dirac—who guessed nature’s secrets by dreaming dreams of mathematical beauty. Their new paradigms were created out of abstract ideas. In those revolutionary years from 1900 to 1930, ideas led the way to understanding.

Peter Galison published in 1997 a fatter but equally illuminating book, *Image and Logic*. Galison gives us a different view of history. He was an experimental physicist before he became a historian. His history is dominated by tools as Kuhn’s was dominated by ideas. Galison does not contradict Kuhn. He quotes Kuhn from time to time with approval. But there is hardly any overlap between the two accounts. The main theme of Galison’s book is the transition from the analog world of cloud chambers and bubble chambers to the digital world of counters and computers in the history of 20th-century particle physics. Analog devices producing pictures were superseded by digital devices producing numerical data. This transition was a profound change. It occurred not only in particle physics but also in other sciences such as geology, meteorology, paleontology, and genetics. Perhaps astronomy is the last remaining science that still has its main tools producing output in the form of images. Even in astronomy, though,



digital output is rapidly gaining ground. The tools of digital data-processing have driven progress in almost all the sciences during the second half of the 20th century. Broadly speaking, the first half of the 20th century belonged to Kuhn and the second half to Galison. Kuhn and Galison are both excellent historians, but each of them depicts a partial view of science. We need both of them to give us a complete picture.

Kuhn’s book covers 500 years from Copernicus to the present. Galison covers less than a hundred. Kuhn gives us vivid accounts of idea-driven revolutions before the 20th century, especially the revolutions associated with the names of Galileo in physics and Lavoisier in chemistry. In those earlier centuries, Galisonian science was already important. The tools of steam-engine technology came first, before the ideas of thermodynamics. The tools of telegraphy and telephony came first, before the ideas of information theory. Kuhn was well aware that Galisonian science existed, but he dismissed it with the epithet “normal.” Kuhn concentrated his attention on scientific revolutions. Normal science was by definition not revolutionary and therefore not interesting.

At the midpoint of the 20th century, when I was a student, the two books had not yet been written, but the world of physics was sharply split into Kuhnian and Galisonian programs. The old heroes of the pre-war revolutions were pursuing private dreams of

The physical sciences have alternated between revolutions driven by new ideas and explorations driven by new tools.

Kuhnian revolutions still to come. Einstein dreamed of a unified field theory. Heisenberg and Schrödinger and Dirac each had a dream based on equations rather than on experiments. Each of them believed that progress in physics could only come through revolutionary new ideas. Each of them dreamed of repeating the triumphs of the 1920s. Meanwhile, the younger generation was using the new tools generated by military technology to push science ahead in Galisonian style. Martin Ryle at Cambridge University was salvaging abandoned military radar dishes and converting them into radio telescopes to explore the universe. He found an astonishing abundance of powerful radio sources at cosmological distances. Willis Lamb at Columbia University was using microwave spectroscopy to explore the fine structure of the hydrogen atom with vastly improved precision. He found evidence for interaction of the atom with charges and currents induced by it in empty space. Maurice Wilkins and Rosalind Franklin at Kings College, London, were using x-ray diffraction to explore the structure of DNA. Their pictures led Francis Crick and James Watson to the discovery of the DNA double helix. Melvin Calvin in Berkeley was using radioactive tracer chemistry and paper chromatography to explore photosynthesis. He found the chain of reactions by which plants use sunlight to convert carbon dioxide into sugar. Four new tools created four new sciences. Ten years after World War II ended, Galisonian science was roaring ahead while Kuhnian dreams had faded. And so it continued for the rest of the 20th century.

At the beginning of the 21st century, we find ourselves in a situation reminiscent of the 1950s. Once again, the community of physicists is split into Kuhnians and Galisonians. The most ambitious of the Kuhnian programs is string theory, building a grand and beautiful structure out of abstract mathematics and hoping to find it somehow mirrored in the architecture of the universe. This program is not an isolated one-man show like Einstein’s unified field theory. String theory is a collective enterprise combining the efforts of thousands of people in hundreds of universities. These people are the best and the brightest of their generation, most of them young and

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many of them brilliant. Their work is admired by the pure mathematicians who share their ideas and speak their language. String theory, as a solid part of modern mathematics, is here to stay. But meanwhile, Galisonian science is continuing to forge ahead, exploring nature without paying attention to string theory. The great recent discoveries in the physical sciences were dark matter and dark energy, two mysterious monsters together constituting 97% of the mass of the universe. These discoveries did not give rise to new paradigms.

We cannot build paradigms out of ignorance. The monsters were discovered by using the new tools of astronomy, wide-field cameras, and digital data processing. We must study the monsters patiently with new and more precise digital tools before we can begin to understand them. Galisonian science will continue to explore, with constantly evolving tools, the structures of space and time and galaxies and particles and genomes and brains.

We are standing now as we stood in the 1950s, between a Kuhnian dream of sudden

illumination and a Galisonian reality of laborious exploring. On one side are string theory and speculations about multiverses; on the other are all-sky surveys and observations of real black holes. The balance today is more even than it was in the 1950s. String theory is a far more promising venture than Einstein's unified field theory. Kuhn and Galison are running neck and neck in the race for glory. We are lucky to live in a time when both are going strong.

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HISTORY OF SCIENCE

The Revolution in the Life Sciences

Sydney Brenner

Historians have the luxury of looking back at human endeavor over long periods of time, but most scientists are too busy working in the present and thinking anxiously about the future and have no time to view their work in the context of what has gone before. I once remarked that all graduate students in biology divide history into two epochs: the past 2 years and everything else before that, where Archimedes, Newton, Darwin, Mendel—even Watson and Crick—inhabit a time-compressed universe as uneasy contemporaries. It seems remarkable that historians once thought that science progressed by the steady addition of knowledge, building the edifice of scientific truth, brick by brick. In his 1962 book *The Structure of Scientific Revolutions*, Thomas Kuhn argued that progress occurs in revolutionary steps by the introduction of new paradigms, which may be new theories—new ways of looking at the world—or new technical methods that enhance observation and analysis.

Between Kuhn's revolutions, scientific knowledge does advance by accretion, as there is much to do to consolidate the new science. But then, inevitably, unsolved problems accumulate and, in many cases, the inconsistencies have been put to one side and everybody hopes that they will quietly go away. The edifice becomes rickety; some of its foundations are insecure and many of the bricks have not been well-baked. This is when a new revolutionary wave in the form of new ideas or new techniques appears, which allows us to condemn and demolish the unsafe or corrupt



parts of the edifice and rebuild truth. Often there is great resistance to the new wave, but as Max Planck pointed out, it succeeds because the opponents grow old and die. The process is then repeated: The radicals become liberals, the liberals become conservatives, the conservatives become reactionaries, and the reactionaries disappear. Students of evolution will recognize this process in the theory of punctuated equilibrium: Organisms stay much the same for very long periods of time; this is interrupted by bursts of change when novelty appears, followed again by stasis.

The life sciences have undergone a radical revolution in my lifetime, and it is interesting to view this from the vantage point of the present to understand its full meaning and impact. In the first half of the 20th century, physics underwent two revolutions: Einstein's theory of relativity, connected with large scales of time and space, and quantum mechanics, concerned with the very small and dealing with fundamental questions of matter and energy. Although Newton still reigned supreme in the human-scale world, the revolutions opened

Recognition of DNA as the carrier of information created a new fundamental dimension for viewing the natural world.

up totally new fields of the physical sciences whose impact continues today. In the meantime, genetics had shown that chromosomes were the carriers of genes that specified the phenotypic characteristics of organisms and were the modern version of the factors postulated by Mendel—but beyond that, very little was known about the material basis of genes and how they accomplished their proposed functions in living organisms. This property attracted the attention of theoretical physicists; one, Schrödinger, wrote a book (*What Is Life?*) that speculated on the physical nature of the genetic material. Many of my contemporaries read this book and claimed it had a great influence on them. I read it but did not understand it, largely because I did not know what Schrödinger meant by an aperiodic crystal. Later, I came to realize that he had made a profound error when he claimed that the chromosomes not only contained the plan for the development of the organism but also had the means to execute it.

Chromosomes were known to contain both DNA and proteins, but many biologists did not believe that DNA could be the carrier of genetic information because its chemical structure was too simple; they thought proteins had to be involved. Avery's demonstration that DNA was the transforming principle of *Pneumococcus* was ignored by most biochemists, but in 1953, the discovery of the double-helical structure of DNA by Watson and Crick changed everything. Very little happened in the first few years, but by 1956, the new molecular biology began to gather momentum, and the rest of the story is well known.

We can now see exactly what constituted the new paradigm in the life sci-

ences: It was the introduction of the idea of information and its physical embodiment in DNA sequences of four different bases. Thus, although the components of DNA are simple chemicals, the complexity that can be generated by different sequences is enormous. In 1953, biochemists were preoccupied only with questions of matter and energy, but now they had to add information. In the study of protein synthesis, most biochemists were concerned with the source of energy for the synthesis of the peptide bond; a few wrote about the “patternization” problem. For molecular biologists, the problem was how one sequence of four nucleotides encoded another sequence of 20 amino acids. It should now be evident what is needed to add to physics to account for living systems. The fundamental theory was formulated by Turing in his notion of a universal Turing machine and deployed by von

Neumann in his theory of self-reproducing machines. Given a description of any computation, a universal Turing machine can read the description and perform the computation; in the same way, a von Neumann universal constructor can build any machine when provided with its description, but to preserve the self-reproducing property, it is necessary for the parent machine to copy its description and insert a copy into the progeny machine. We can now recognize Schrödinger’s mistake: The chromosomes do not contain the means for executing the plan of the organism, but only a description of the means. There are no causal relationships between Turing’s and von Neumann’s ideas and those of Watson and Crick. They got their ideas of the genetic code from common parlance; Francis Crick once told me that he saw it like the Morse code—as a table transforming the alphabet of letters into the

binary code of dots and dashes. The connections exist only in the plane of the history of ideas.

We also can provide the answer for those physicists who looked for new laws of physics in biology: Biology is essentially (very low energy) physics with computation. Fundamental theory in biology is concerned principally with viewing living organisms as the only part of the natural world whose members contain internal descriptions of themselves. That is why I could once tell a Buddhist priest that mountains were not alive: You can’t clone a mountain. It is also why the whole of biology must be rooted in DNA, and our task is still to discover how these DNA sequences arose in evolution and how they are interpreted to build the diversity of the living world. Physics was once called natural philosophy; perhaps we should call biology “natural engineering.”

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EVOLUTION

Who Speaks with a Forked Tongue?

Jonathan B. Losos,¹ David M. Hillis,² Harry W. Greene³

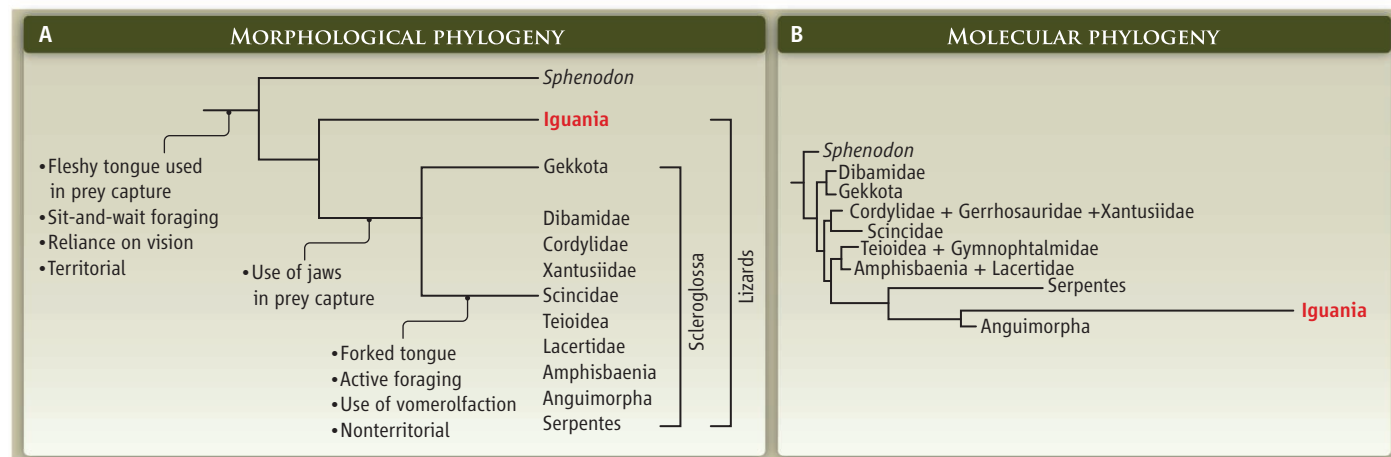
At the dawn of molecular phylogenetics, much was made of the conflict between results from morphological and molecular data sets. Although molecu-

lar data have rarely changed our understanding of the major multicellular groups of the evolutionary tree of life, they have suggested changes in the relationships within many groups, such as the evolutionary position of whales in the clade of even-toed ungulates (1). Further investigation has usually resolved conflicts, often by revealing inadequacies in previous morphological studies. This has led to a presumption by many in favor of molecular data, but a recent morphological analysis

by Gauthier *et al.* (2) argues persuasively that we should reconsider whether DNA is always inherently superior for inferring life’s history.

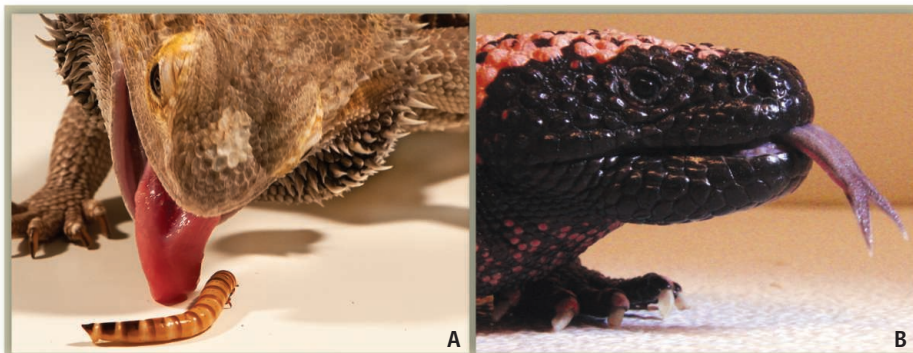
Molecular analysis has clear advantages. Vast quantities of sequence data can be collected rapidly and at ever-lower cost; the sequences can be scored objectively and repeatedly; they often are not as prone to misleading adaptive convergence, and avoid problems of environmentally induced variation. Nonetheless, new molecular phylog-

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Evolutionary conundrum. (A) In the morphology-based phylogeny, many key characters in iguanians are inferred to represent the retained ancestral state. *Sphenodon* is the closest living relative to lizards. [Phylogeny based on (14)] (B) In the

molecular phylogeny, iguanians are placed high in the tree; their supposedly ancestral characters are attributed to evolutionary reversals. Branch lengths are proportional to the number of morphological reversals required by this tree.



Evolutionary reversal or ancestral state? One of the traits inferred to have reversed evolutionarily in the lizard molecular tree is the ancestral tongue morphology, shown here for the iguanian *Pogona barbatus* (A); the scleroglossan *Heloderma suspectum* (B) exhibits the derived, bifid state.

enies are not always correct. A 1991 study concluded that guinea pigs are not rodents (3), but higher sampling of taxa revealed a methodological bias and showed that they are indeed rodents (4). In a similar vein, errors in rooting molecular trees have led to mistaken conclusions, such as that toothed whales evolved multiple times (5). Other problems with molecular analyses stem from overly simplistic substitution models that fail to account for details of genome evolution (6), confusion arising from gene duplication (7), and missing genes (8). None of these exemplify inherent problems with molecular data but rather false assumptions about how genomes evolve. A study is not necessarily better just because it uses DNA analysis (9).

And that brings us to lizards. The standard view has been that the ~9000 lizard species split at the base of the phylogeny into Iguania (iguanas, chameleons, and relatives) and Scleroglossa (all remaining lizards, including geckos, skinks, monitors, and snakes) (see the first figure, panel A). The straightforward evolutionary scenario from this phylogeny was that iguanians exhibit many ancestral characteristics and that the evolution of scleroglossans reflected a suite of derived and often concerted changes.

In the past decade, molecular phylogenetic analyses, culminating in Wiens *et al.*'s study (10), have strongly contradicted this view. Most surprisingly, they find that iguanians evolved more recently, nesting high in the lizard tree close to monitors and Gila monsters (Anguimorpha) and snakes (Serpentes) (see the first figure, panel B). In this view, supposedly ancestral characteristics of iguanians arose because they re-evolved character states shared with more distant relatives but not seen in snakes, monitors, and others among their newfound phylogenetic neighbors (see the second figure).

These findings did not sit well with Gauthier's team of morphologists, who have

built an enormous data set, examining 192 species of extant and extinct lizards and 610 variable characters, 247 of them previously unrecognized and most only now accessible through high-resolution x-ray computed tomography. Many systematists likely expected that a greatly enhanced morphological data set, analyzed with state-of-the-art phylogenetic methods, would echo DNA-based studies, but the results could hardly have been more contradictory. The analysis overwhelming supports the traditional phylogeny; not a single anatomical synapomorphy (a shared, derived character that suggests close relationship) supports placement of Iguania high in the lizard tree. Moreover, an enormous number of evolutionary reversals—traits evolving back to the ancestral lizard condition—is required on the branch leading to the Iguania.

When two phylogenies are fundamentally discordant, at least one data set must be misleading. There are two plausible explanations for this conflict. One is that morphological homoplasy [multiple evolution of character states by convergent evolution or reversal (11)] is rampant, falsely signaling that iguanians possess a remarkable number of ancestral character states and incorrectly placing them at the base of the lizard tree. Gauthier *et al.* regard this as unlikely, because the synapomorphies of scleroglossans inferred as lost by iguanians in the molecular tree come from many functionally different parts of anatomy. These traits have disparate embryological origins and growth patterns, discounting general explanations based on development. Furthermore, iguanians have diverse lifestyles, ranging from large herbivorous iguanas to ant-eating horned lizards and gliding dragons. It is hard to see how this multifaceted suite of characteristics could reflect adaptation to an overall iguanian lifestyle.

Could the explanation be that the molecular data are providing the false signal? Nat-

ural selection operates at molecular as well as morphological levels, and examples of molecular convergence confounding phylogeny bear out this concern (12). Moreover, differential selection for base composition or particular codon usage could produce biased patterns of genetic evolution, skewing a phylogenetic analysis (13). But what processes are sufficient for the 44 protein-coding genes analyzed by Wiens *et al.* (10) to produce a consistent bias and so radically restructure the lizard tree? Higher rates of molecular evolution in iguanians and snakes (1) suggest that the genes in these taxa are not evolving like those in other lizard lineages, but it is unclear how this rate heterogeneity might violate assumptions of the underlying models used to infer the molecular phylogeny.

We are left with a conundrum. The molecular data imply an astonishing pattern of morphological homoplasy and suggest very limited knowledge of the functional link between structures and lifestyle; if convergence is so pervasive, what faith can we have in the placement of fossil taxa for which no molecular data are available? Conversely, morphology implies a pattern of molecular evolution that has yet to be explained.

Beyond this intriguing discrepancy, Gauthier *et al.*'s analysis shows the continuing power and importance of morphological and fossil investigation. Lizards are surely not special in harboring so much undescribed and little understood variation, and state-of-the-art technological tools promise revelations never previously imagined.

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MOLECULAR BIOLOGY

EZH2 Goes Solo

Giacomo Cavalli

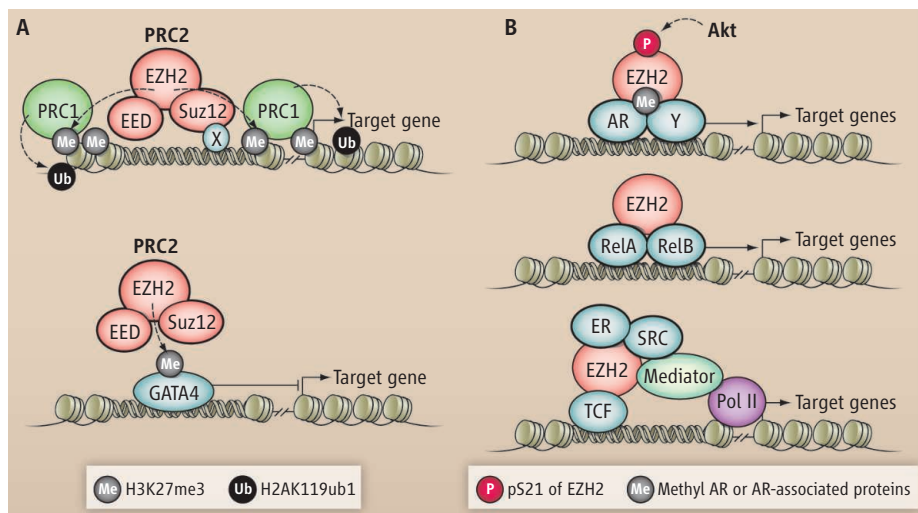
Enhancer of zeste homolog 2 (EZH2), the catalytic subunit of Polycomb repressive complex 2 (PRC2), silences transcription through trimethylation of histone H3 lysine 27 (H3K27me₃). EZH2 is frequently overexpressed in many cancer types, including breast, lung, and prostate cancer. EZH2 overexpression is also linked to poor survival, making it an important target in cancer therapy (1, 2). Prostate cancer can be treated by removal of testicular androgens, but androgen depletion is frequently associated with the recurrence of malignant neoplasms, defined as castration-resistant prostate cancer (CRPC), which has a poor prognosis. In prostate cancer, EZH2 represses the two tumor suppressor genes *ADRB2* and *DAB2IP* (3, 4). However, on page 1465 of this issue, Xu *et al.* analyze the role of EZH2 in CRPC and report that the oncogenic function of EZH2 does not depend on gene silencing, but rather on transcriptional induction of a subset of its target genes (5).

As a model for CRPC, the authors used the androgen-independent LNCaP-abl (abl) prostate cancer cell line and the parental LNCaP androgen-dependent line (6). Although EZH2, but not other PRC2 subunits, is overexpressed in abl cells, the authors found that the EZH2-associated H3K27me₃ is lower than in LNCaP cells. Xu *et al.* then investigated the genome-wide location of EZH2 and found that a subset of EZH2-bound genes did not bind the PRC2 subunit SUZ12 or display H3K27me₃. Many of these “solo” genes were down-regulated upon EZH2 knock-down, arguing for a PRC2-independent role of EZH2 in their activation.

EZH2 generally catalyzes trimethylation of H3K27 via its SET domain (7). Although H3K27me₃ is not deposited at EZH2-induced solo genes, Xu *et al.* show that the methyltransferase activity of EZH2 is required for both EZH2-dependent gene activation and androgen-independent growth. This suggests that other factors may be methylated by EZH2 to mediate its activity. Indeed, previous studies have shown that EZH2 can methylate nonhistone proteins, such as the transcription factor GATA4 (8).

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A transcriptional repressor switches to an activator function in an aggressive form of prostate cancer.



Possible mechanisms for EZH2 action. (A) EZH2-mediated gene silencing via recruitment of the PRC2 complex (including EZH2, Suz12, and EED) by DNA-binding proteins (X). Silencing occurs via deposition of H3K27me₃ on chromatin and PRC1 recruitment (top), which also ubiquitinates (Ub) histone H2AK119. Repression via factor methylation may also occur, such as in the case of GATA4 (bottom). (B) EZH2-mediated gene activation. This may be mediated by EZH2 recruitment by the androgen receptor (AR) and methylation of AR or associated (Y) proteins (top), by formation of a ternary complex with the RelA/RelB NF-κB components (middle), or by bridging TCF/β-catenin and ER/SRC-1 with the Mediator complex to induce RNA polymerase II transcription (bottom).

In the case of CRPC, the androgen receptor is a candidate methylation target; Xu *et al.* show that it co-binds with EZH2 at many solo genes and interacts with EZH2. Furthermore, EZH2 depletion does not change androgen receptor levels, but it decreases androgen receptor-associated lysine methylation. The androgen receptor is methylated at lysines 630 and 632, and Set9-mediated methylation of these lysines was proposed to potentiate its transcriptional activity (9, 10). Therefore, a potential mechanism for EZH2-mediated transcriptional activation is via methylation of the androgen receptor, although methylation of other androgen receptor-associated proteins is also possible (see the figure).

What drives EZH2 to interact with the androgen receptor in CRPC cells? A good candidate that was explored by Xu *et al.* is phosphorylation at serine 21, which can be mediated by the Akt pathway (11–13). Indeed, elevated levels of Akt and of phosphorylated Ser²¹ (pS21) EZH2 were found in abl cells as well as in other hormone-refractory prostate cancer cell lines. pS21 EZH2 locates preferentially in “solo” genes, and an EZH2 mutant abolishing Ser²¹ phosphor-

ylation could neither induce expression of “solo” genes nor support androgen-independent growth of CRPC cells. These data support the view that Akt activation in CRPC cells phosphorylates Ser²¹ of EZH2 to suppress H3K27me₃, whereas the binding of pS21 EZH2 stimulates methylation of the androgen receptor and transcriptional activation at “solo” genes.

The finding of an activator role for EZH2 is not unprecedented. In breast cancer cells, EZH2 can switch from a repressor into an activator in two different ways (14, 15). In luminal-like estrogen receptor (ER)-positive cells, EZH2 overexpression can lead to its interaction with the Wnt signaling components TCF and β-catenin as well as with the ER, and leads to activation of the *c-myc* and *Cyclin D1* genes (15). In basal-like, ER-negative cells, EZH2 activates NF-κB target genes by formation of a ternary complex with the NF-κB components RelA and RelB (14). In both cases, EZH2 functions without the other PRC2 components, but the activation mechanism differs from the one found by Xu *et al.* because it does not require an intact SET domain (14, 15). Therefore, overexpression of EZH2 may free excess protein

from PRC2 complexes, enabling binding to other proteins and, at least in some cases, to activation of target genes. Activating roles for other Polycomb proteins have also been suggested (16–18). Future studies should be directed at uncovering the circumstances under which Polycomb factors switch from their canonical repressor function to that of transcriptional coactivators.

These data have implications that extend beyond cell lines. Indeed, Xu *et al.* show that in cohorts of CRPC patients overexpressing EZH2, EZH2-stimulated solo genes are frequently up-regulated, and their higher expression levels correlate with poor survival. Finally, the identification of EZH2 phosphor-

ylation as an inducer of EZH2-mediated gene activation via interaction with the androgen receptor opens new therapeutic avenues targeting the activation function of EZH2 without affecting H3K27me3. It remains to be seen whether these new strategies will extend to other cancer types.

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GENETICS

A Genetic Intervention Stands a Skip Away from Clinical Tests

Jeffrey S. Chamberlain

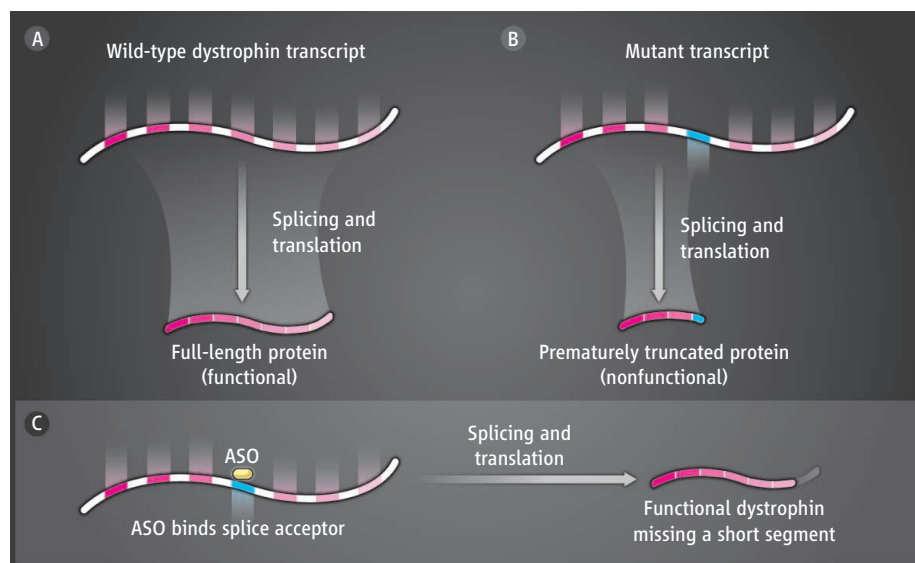
Establishing dystrophin as the mutated gene in Duchenne muscular dystrophy (DMD) was arguably the first successful use of genetic information to identify a human disease gene (1). Despite the large investment in the Human Genome Project in the ensuing quarter-century, few therapies have been developed for genetic disorders. Fortunately, this is starting to change for DMD, a condition characterized by loss of functional muscle fibers and progressive muscle weakness and deterioration. Multiple therapies, including gene therapy, gene up-regulation, and transcript modification, are in various stages of clinical trials for the disease. One promising approach uses antisense oligonucleotides to induce a process called exon skipping in precursor mRNA (pre-mRNA), and has advanced to phase II clinical trials (2, 3). However, results thus far are below the threshold needed for the therapy to be effective. Kendall *et al.* now report a class of drugs that increases the efficiency of exon skipping (4). This could prove critical to achieving therapeutic benefit and increases the prospects for using antisense oligonucleotides as a treatment for DMD.

The rationale for exon skipping is based

on patients carrying deletions of one or more exons (mRNA coding regions) of the dystrophin gene. Some of these mutant genes produce shortened dystrophin proteins that are highly functional in muscle (5, 6). Dystrophin is part of a protein complex that connects the cytoskeleton of a muscle fiber to the cell membrane. Individuals with exon deletions

A drug that improves production of dystrophin protein reduces disease severity in a mouse model of Duchenne muscular dystrophy.

in the dystrophin gene are sometimes asymptomatic but more typically display a mild phenotype known as Becker muscular dystrophy. The effectiveness of shortened dystrophins depends on their stability, the part of the protein that is removed, and the phasing of repeat units that form the major part of the protein; surprisingly, the exact size of the



Exon skipping in DMD. (A) Schematic of the wild-type dystrophin transcript (pre-mRNA) with exons (pink) and introns (white) shown. The pre-mRNA is spliced and translated into the dystrophin protein. (B) With mutant dystrophin genes, individual exons may be deleted or contain mutations [such as nonsense codons (blue)] that prevent dystrophin expression. (C) An antisense oligonucleotide (ASO) that targets splice donors, for example, can induce exon skipping such that a functional protein that is missing a small segment is translated.

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deletion is less important (7–9). By contrast, mutations found in DMD patients prevent production of dystrophin. However, in many cases, a functional protein can be encoded if one or more exons are removed from the pre-mRNA, allowing the production of a shortened and partly functional dystrophin (see the figure). This exon skipping can be triggered by the presence of antisense oligonucleotides that anneal with splice donors, splice acceptors, or splicing enhancers located in exons of the pre-mRNA.

Several oligonucleotide chemistries have been tested for this purpose, with a goal of finding nucleotide backbones that are stable, nontoxic, and display a high affinity for RNA (10). The two most promising chemistries, now in early-phase clinical trials, induce a substantial amount of exon skipping in muscles from a mouse model of DMD (2, 3, 11). In both cases, dystrophin production has been observed by immunofluorescence in muscle sections and biochemically from muscle biopsies. Importantly, this restoration of dystrophin can be achieved when the antisense oligonucleotides are delivered systemically by subcutaneous or intravenous injection.

However, several limitations of the technology may constrain its overall impact. Dystrophin production induced by antisense oligonucleotides has not yet achieved the amounts needed for a robust therapy. Also, the efficiency of exon skipping varies widely from one muscle group to another and between patients. Studies in mice and humans suggest that 20 to 30% of normal dystrophin production is needed for an effective treatment, a level seen only in some muscles of patients receiving the highest dose of antisense oligonucleotides (12, 13). Also, although some muscle fibers produce high amounts of dystrophin, others make little, so the 20 to 30% range is an average across an entire muscle. The reasons for these variations are unclear but may relate to barriers to cell entry or to accessibility of the antisense oligonucleotides to the cell's mRNA splicing machinery. Another limitation is that different antisense oligonucleotides must be developed for different exons in the dystrophin gene. Ongoing clinical trials use antisense oligonucleotides that target exon 51, which could be beneficial for ~13% of all patients (14). In addition, current antisense oligonucleotide chemistries are not effective in the heart, yet many DMD patients die of cardiac complications. There are also concerns about long-term toxicity, as antisense oligonucleotides must be administered frequently.

Kendall *et al.* address several of these limitations by identifying a drug that enhances

the efficiency of exon skipping. The drug, dantrolene, acts on the ryanodine receptor, which regulates cellular calcium signaling in the cytoplasm and the nucleus. Other antagonists of the ryanodine receptor also enhanced exon skipping, although dantrolene was the best. These agents, independently, did not affect muscle pathology, which suggests that each mechanism of action is directly coupled to pre-mRNA splicing that is mediated by antisense oligonucleotides. Dantrolene is approved by the U.S. Food and Drug Administration and has been tested in a DMD clinical trial without adverse events (15). Thus, the drug appears ready for clinical testing with antisense oligonucleotides.

Several features of the study by Kendall *et al.* suggest that even more efficient enhancers of skipping might be identified. Dantrolene was found after screening relatively few compounds, yet the screening protocol is amenable to considerable scale-up. It will be important to identify the mechanism by which the ryanodine receptor antagonists work, because doing so may allow rational design of more potent compounds. Do calcium concentrations directly modulate the cell's splicing machinery in a manner that enhances exon skipping, or might antisense oligonucleotides be rendered ineffective in the presence of elevated calcium?

Uncovering this answer might also lead to exon-skipping enhancers that work in cardiac muscle. One concern is that dantrolene was only tested with suboptimal concentrations of antisense oligonucleotides (to simplify the identification of an exon-skipping enhancement); it will be critical to determine whether these compounds are also effective at the higher doses used in the clinic. Although the wait has been long, the era of direct genetic intervention to reduce the severity of DMD is getting closer, and it is reasonable to assume that similar approaches will be useful for other genetic disorders.

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CHEMISTRY

Relay Reactions That Trap Organometallic Intermediates

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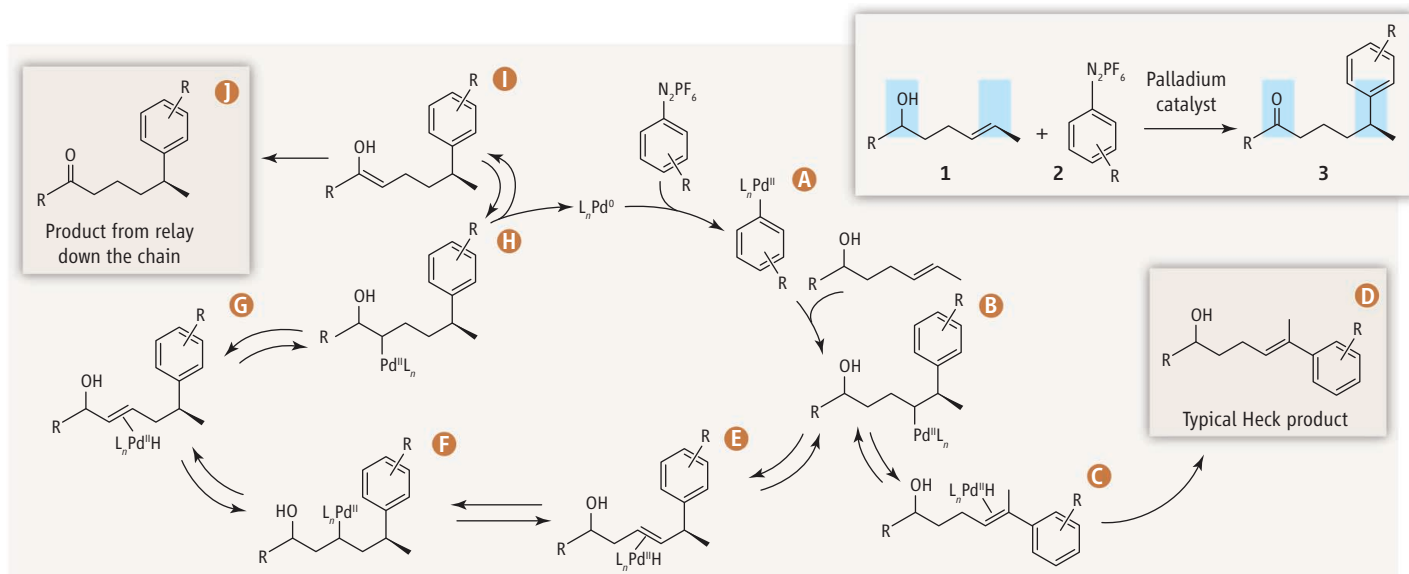
The movement of a palladium catalyst for a Heck reaction ultimately creates a ketone group far from the site of carbon-carbon bond formation.

In 1968, Richard Heck reported (in seven remarkable consecutive papers) on palladium addition and coupling reactions that create new carbon-carbon bonds (1–7). These reactions and many variants became known as the Mizoroki-Heck reaction, and their use in chemical synthesis (especially of complex natural products) greatly reduced the number of reaction steps needed compared to traditional uncatalyzed reactions. Through the careful use of different transition metals, substrate partners, reaction conditions, and ligands coordinated to the transition metal,

chemists can now control product formation, both where a reagent reacts on a molecule (regiochemistry) and how it reacts in a three-dimensional sense (stereochemistry). What has been rare is to combine the formation of a desired bond with the refunctionalization of the molecule at a remote site. On page 1455 of this issue, Werner *et al.* (8) report a stereoselective Heck reaction in which not only are the regiochemistry and stereochemistry of the reaction controlled, but a ketone is formed several bonds away through a series of relay of reactions down the substrate chain.

Many classic organic reactions that form carbon-carbon bonds result in products that have less functionality than the starting mate-

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rial. This approach often results in the need for extra synthetic steps to refunctionalize the molecule so additional “productive” reactions can be carried out, and leads to low efficiency and overall yields. Also, when chemical reactions are run on one section of a molecule, the remainder of the structure is often carried along unchanged (which is also inefficient if it requires further modification).

Additions to carbon-carbon double bonds are typically controlled by steric or electronic factors that determine which of the two carbons a reagent will favor. Werner *et al.* used a transition metal and ligand combination that not only controlled carbon stereochemistry and regiochemistry, but allowed for the use of mild reaction conditions that preserve sensitive reaction intermediates. The lower reaction temperature presumably allowed the organometallic reaction intermediates to transmit their functionality down the molecule. Although palladium is commonly used for such reactions, the nitrogen-based bidentate ligand is not. This ligand system was selected because of the desire to run the reaction with aryldiazonium salts. These allow for the use of lower reaction temperatures but have not been used because of their incompatibility with the most common ligands for the palladium-catalyzed Heck reactions, phosphines.

Having recognized precedent for this type of intermediate migration (9–11), Werner *et al.* had to address a number of issues to make the process general. The replacement of the typical aryl halide with a diazoarene would provide milder reaction conditions, but the typical phosphine ligands for stereoselective Heck reactions would not be amenable, given their incompatibility with this functionality. This limitation required

the development of nitrogen-based ligands, although none were known to perform this reaction in the regio- and stereoselective manner desired. The authors capitalized on their previous experience with a multidimensional parameter-based optimization protocol to find the necessary ligand (12). This process was facilitated by the selection of ligand systems that are modular in nature so that a variety of structures were accessible for their optimization protocol.

As illustrated in the figure, the first step of the catalytic cycle for this Heck reaction is a room-temperature oxidative addition to form the aryl palladium **A**. After addition to the olefin **B**, the alkyl palladium abstracts a β -hydrogen to give an olefin complex **C**. Under most Heck reaction conditions, this represents the end of the cycle **D**—the metal would return for another oxidative addition to an aryl halide.

Under the milder conditions reported by Werner *et al.*, the β -hydride elimination appears to be reversible, so reformation of the alkyl palladium can be followed by β -hydride elimination away from the site of addition to give palladium olefin complex **E**. Palladium can then re-add to the olefin but from the opposite ends **F**, effectively moving the metal down the chain. The subsequent β -hydride elimination away from the original site of addition then moves the double bond down the chain. These additions and eliminations likely take place in both directions until a β -hydride elimination takes place that gives an enol form of a ketone **I**. This reaction is formally an oxidation of the alcohol **H**. The subsequent tautomerization (migration of the H from OH to carbon) then results in a carbonyl that ends the relay, trapping the product as the ketone **J**.

Keep moving. A catalytic reaction at the double bond of compound **1** (upper right) reported by Werner *et al.* formed a new carbon-carbon bond and oxidized a distant alcohol group to a ketone (blue boxes); R is an organic group and L is a ligand. The catalytic cycle (main panel), involving a series of β -hydride eliminations followed by migratory insertions, moves the palladium center up and down the chain until the formation of an enol and its tautomerization to a ketone **J** stops the process.

Organometallic chemistry offers the opportunity to functionalize molecules in ways that were not previously possible. The work of Werner *et al.* illustrates how regio- and stereochemistry can be controlled by the optimization of ligand sets around metals, and after the initial reaction, the resulting organometallic species can be used to relay their functionality to sites remote from the initial location of reaction. Additionally, their report illustrates the power of their multidimensional parameter-based optimization protocol that allowed them to rapidly find an appropriate ligand set to carry out the reaction in a stereoselective manner.

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CHEMISTRY

Sub-Nanosized Gold Catalysts

A. Stephen K. Hashmi

Gold was long considered to be “catalytically dead” (1), but since 1985, triggered by observations of Haruta (2) and Hutchings (3), heterogeneous gold catalysis has become a highly active field. Innovative results from homogeneous gold catalysis have also triggered considerable research interest in this area (4). These developments have mainly been of academic interest; investigations into new gold catalysts, reactions, and mechanisms have in most cases been conducted with relatively high catalyst loadings of ~5 mol% and small amounts of substrate (100 to 200 mg), and only a few applications in the synthesis of fine chemicals are known. On page 1452 of this issue, Oliver-Meseguer *et al.* (5) report that small gold clusters of only 3 to 10 atoms can efficiently catalyze the addition of water to alkynes at catalyst loadings lower than anyone has reported before. The results will open the door for future industrial applications beyond fine chemicals, which are typically produced in smaller scale.

The crucial value describing catalytic activity is the number of substrate molecules that one catalyst molecule can convert to the product before some deactivation event takes place; this number is called the turnover number (TON). This competition between catalysis and deactivation obviously also has a time component. The speed at which the catalyst operates is characterized by the number of substrate molecules converted per time unit. This number is called the turnover frequency (TOF) and gives a measure of the reactor size needed for industrial production.

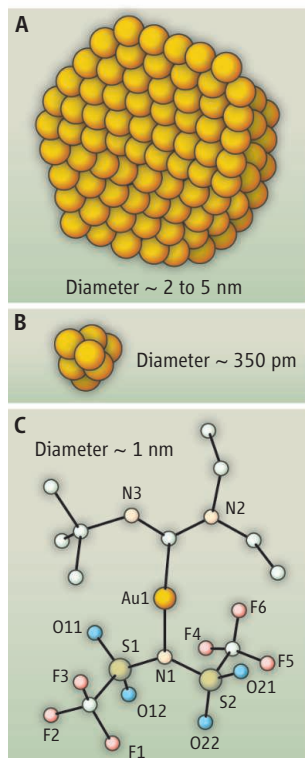
In the case of a catalyst loading of 5 mol%, a yield of 100% corresponds to a TON of 20—a number that is ridiculously low in the eyes of any industrial chemist. In gold catalysis there are only a few examples of higher turnover numbers. Teles *et al.* (6) have explored the homogeneous gold(I)-catalyzed addition of alcohols to alkynes on a multi-kilogram scale and achieved an impressive TON of 100,000 and a TOF of 5400 hour⁻¹. Through the use of CO and acid as promoters and cocatalysts in this homogeneous catalysis, Mizushima *et al.* (7) subsequently almost tripled this TOF to 15,600 hour⁻¹. For the hetero-

geneous gold-catalyzed oxidation of glucose by oxygen, Comotti *et al.* (8) achieved an even higher TOF of 50,120 hour⁻¹. Finally, Marion *et al.* recently reported a homogeneous and highly efficient hydration of alkynes, reaching a TON of 84,000 and a TOF of 4667 hour⁻¹ with some substrates (9).

Examples of highly efficient heterogeneous gold catalysts also stem from the field of alcohol addition to alkynes; last year Bouhrara *et al.* reported a TON of 800,000 and a TOF of 294,000 hour⁻¹ (10). These water or alcohol additions to alkynes seem to be by far the most efficient gold-catalyzed reactions; other reactions with more complex mechanisms and more delicate intermediates have much lower TONs. For example, the gold-catalyzed phenol synthesis was initially reported with a TON of 48 (11); a TON of 3050 (12) remains the best value known. For the cyclization of allenyl ketones to furan heterocycles, Zhou *et al.* reported a TON of 8300 and a TOF of 1700 hour⁻¹ (13).

Oliver-Meseguer *et al.* have now achieved remarkable values of TON (10,000,000) and TOF (100,000 hour⁻¹) for the ester-assisted hydration of alkynes. The key to this success was the use of small gold clusters, which the authors call “sub-nanosized” (see the figure). Such species have barely been explored with regard to their application in catalysis (14, 15). The clusters were formed in situ by adding the catalysis substrate to a solution of AuCl or HAuCl₄ in the absence of stabilizing ligands. They were detected by MALDI mass spectrometry and ultraviolet spectroscopy, and the catalytic activity was correlated to the species present. Because the clusters are not attached to a support, this work falls between heterogeneous and homogeneous catalysis. It is important both for scientists working on nanoparticles and for researchers in the field of gold catalysis, especially those interested in industrial chemistry.

Small gold clusters catalyze water addition to alkynes at the high efficiencies required for bulk industrial applications.



Gold catalysis. Oliver-Meseguer *et al.*'s sub-nanosized gold clusters (B) show catalytic activities that are much higher than those obtained with small gold nanoparticles (A) and mononuclear homogeneous gold catalysts (C).

Oliver-Meseguer *et al.*'s observation is in contrast to Haruta's pioneering work on carbon monoxide oxidation by nanoparticles 2 to 5 nm in diameter (2). Oliver-Meseguer *et al.* study an intramolecular reaction of a substrate molecule, whereas Haruta investigated an intermolecular reaction of two different molecules reacting on a support containing gold nanoparticles.

One deficit of gold catalysis has been the lack of industrial applications in the field of bulk chemicals, which are produced at a large scale. Oliver-Meseguer *et al.*'s TONs are at a level that can match the needs of such processes. The results will inspire others to search for similarly efficient catalyst systems for other reaction types known to be catalyzed by gold or other transition metals. They will also inspire scientists working with other metals to transfer the principles of sub-nanosized catalytic particles to their systems.

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Epigenetic Regulation by Long Noncoding RNAs

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Recent studies show that transcription of the mammalian genome is not only pervasive but also enormously complex. It is estimated that an average of 10 transcription units, the vast majority of which make long noncoding RNAs (lncRNAs), may overlap each traditional coding gene. These lncRNAs include not only antisense, intronic, and intergenic transcripts but also pseudogenes and retrotransposons. Do they universally have function, or are they merely transcriptional by-products of conventional coding genes? A glimpse into the molecular biology of multiple emerging lncRNA systems reveals the “Wild West” landscape of their functions and mechanisms and the key problems to solve in the years ahead toward understanding these intriguing macromolecules.

RNA has become widely suspected as the culprit behind almost every case of epigenetic regulation. There continues to be a shift in how we conceptualize this remarkably versatile macromolecule, once regarded primarily as mere intermediary of the “central dogma” stating that information moves unidirectionally from DNA to RNA to protein. The latest interests center around the genomic “dark matter” that has for years been dismissed as transcriptional noise (1–4). Only 1% of the mammalian genome carries protein-coding potential, yet 70 to 90% is transcribed at some point during development to produce a large transcriptome of long noncoding RNA (lncRNA, defined as RNA > 100 nucleotides in length). Some estimate total membership to exceed 200,000, whereas others suggest fewer than 10,000 (5–8). The ENCODE project has revealed an enormous complexity, with ~10 isoforms overlapping any previously annotated genes, thereby challenging the traditional definition of a gene (8). Although there is now little doubt that pervasive transcription occurs, whether this activity is universally functional is unknown. These transcripts are often poorly conserved, unstable, and/or present in few copies (7, 9, 10). Nonetheless, clear roles have emerged for some lncRNAs, and a survey of some examples illustrates how this class of RNA is helping to establish new paradigms for epigenetic regulation.

Lessons from the X Chromosome

The intriguing story of lncRNAs first debuted in the phenomena of genomic imprinting and X-chromosome inactivation (XCI) (11–14). Nowhere is the abundance of lncRNA more evident than the X-inactivation center (*Xic*). To balance X-chromosome gene expression between males and females, the *Xic* on the mammalian X

chromosome controls the initiation steps of XCI through a series of RNA-based switches (13, 14). Today, the *Xic* serves as a model for understanding epigenetic regulation by lncRNA (Fig. 1).

The X-inactive-specific transcript (XIST/Xist) was one of the first lncRNAs to be discovered in mammals (15). The *Xist* locus produces a 17- to 20-kb RNA that coats the X chromosome in cis (Fig. 1, A and B) (16), is expressed only from the inactive X chromosome (Xi), and is essential for the silencing process (14). Its noncoding status immediately implied that RNA itself could be an effector of chromatin and transcriptional change, an idea substantiated years later by the isolation of Xist's first interacting factors, Polycomb repressive complex 2 (PRC2) (17) and YY1 (18). Through a conserved repeat motif (Repeat A, RepA), Xist RNA directly binds PRC2 (17), the epigenetic complex responsible for trimethylation of histone H3 at Lys²⁷ (H3K27me3), and targets PRC2 to the Xi. These findings indicated that lncRNAs may be crucial accessory factors for Polycomb function. Like other regulatory factors, epigenetic complexes must be targeted in space (genomic location) and time (during development), but many, such as PRC2, do not possess sequence-specific DNA-binding subunits to guide them. The involvement of RNA, a macromolecule with inherent sequence information, would at once provide targeting specificity and introduce new regulatory capabilities (e.g., action in cis).

Targeting PRC2, however, is biologically separable from loading onto chromatin (17). The 1.6-kb RepA transcript recruits PRC2 to the *Xist* promoter, but docking of PRC2 is precluded by expression of *Xist*'s antisense transcript, Tsix. Only when Tsix expression is down-regulated during development does the Xist-PRC2 complex load onto the Xi nucleation center within *Xist*'s exon 1. Loading depends on the transcription factor, YY1, bound only to Xi (18) (Fig. 2). By cotranscriptionally tethering Xist RNA to the *Xic*, YY1 bridges the lncRNA and chromatin, accounting

for the allele-specific binding of Xist RNA to the Xi.

Xist is controlled by two other lncRNAs, one acting negatively (Tsix), the other positively (Jpx). Tsix determines allelic choice by repressing *Xist* transcription on one allele (19, 20). From numerous genetic manipulations at *Tsix*, it now appears that *Tsix* regulates *Xist* in several ways. *Tsix* coordinates X-chromosome pairing to generate epigenetic asymmetry within the *Xist* locus (21); it recruits DNA methyltransferase (Dnmt3a) to silence *Xist* (22, 23); and it blocks recruitment of PRC2 to *Xist* by RepA (17). Tsix RNA directly binds PRC2 (17) and also duplexes with Xist-RepA RNA (24), thereby potentially serving as decoy for PRC2 recruitment (by titrating Xist-RepA RNA or PRC2) in its role as repressor of XCI.

Positive regulation of *Xist* involves another lncRNA, Jpx (25) (Fig. 1B). Deleting *Jpx* abolishes *Xist* activation, its post-transcriptional knockdown recapitulates the deletion, and autosomal expression of *Jpx* rescues the X-linked deletion. Thus, *Jpx* is diffusible and acts in trans as an RNA. Combined, these studies demonstrate central functions for lncRNA during XCI: They directly target repressive epigenetic complexes, act as antisense inhibitors, and activate transcription.

Why Long Noncoding RNAs?

Although lncRNAs now dominate the *Xic*, this region was once coding (26). Evolution of random XCI 150 million years ago in eutherian mammals coincided with a shift from coding to noncoding space, suggesting that lncRNAs offer distinct advantages over proteins for some forms of epigenetic regulation. Allelic and cis control were likely major driving forces behind this “reverse evolution” (27), because XCI treats two X chromosomes in diametrically opposite ways and requires coordinated cis-limited silencing of genes on the Xi. Two properties of mammalian lncRNAs are notably relevant. One feature is lncRNA's tethering capabilities and fast turnover, which enable allelic marking. lncRNAs are naturally tethered to the site of transcription through the RNA-DNA-polymerase (Pol II) ternary complex, thereby enabling function as allele-specific tag (Fig. 2). lncRNAs may have an exposed 5' business end to capture chromatin complexes and a nascent 3' tethering end to anchor the RNA-protein complex to a locus. Inclusion of Pol II pausing could transiently stabilize tethering. Tethering could also be enhanced by bridge proteins, such as YY1 (18). Rapid degradation after transcriptional termination would limit the RNA's half-life, thereby preventing diffusion and action at ectopic sites. Tsix's half-life of 30 to 60 min indicates that the 40-kb transcript is degraded as soon as it is created (23), likely explaining its strict cis action, because effective concentrations would only be reached at the site of synthesis. Fast turnover rates and low copy numbers are features of many lncRNAs (7–10). lncRNAs

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are thereby distinguished from proteins and small RNAs by the possibility of allele-specific action. Proteins do not retain allelic memory; their transcriptional origin is lost when mRNA is shuttled to the cytoplasm for translation to protein. Small RNAs of the RNA interference (RNAi) pathway would be ineffective tethers because of their size.

Another property of lncRNAs is their ability to specify a unique address through use of a large sequence space. Transcription factors are effective recruiting factors for epigenetic complexes, but an advantage of lncRNAs is the possibility of targeting to a single location. Transcription factors recognize short DNA motifs that typically occur thousands of times in the genome. Transcription factors therefore necessarily act within large networks and affect hundreds of genes at once. By contrast, lncRNAs like Tsix and RepA/Xist occur only once. This singularity enables delivery of epigenetic complexes to a unique address, offering a regulatory specificity not possible with proteins and small RNAs. Because there are no a priori limits on length and composition, the sequence space for lncRNA-mediated targeting greatly exceeds that of binding motifs for the proteome.

Local and Genome-Wide Control

A collaboration between site-specific lncRNAs and network-based transcription factors together with chromatin modifiers would account for spatial and temporal specificity during development. For example, whereas the transcription factor OCT4 responds to leukemia inhibitory factor and bone morphogenetic protein signaling in pluripotent cells to activate a genome-wide transcription program, local effects may be achieved through OCT4-responsive lncRNAs, such as Miat (28), Xite, Tsix, and Xist (21, 29). At the *Xic*, for instance, OCT4 activates Tsix and Xite and in doing so controls X-chromosome pairing and initiation of the XCI cascade. Tsix RNA in turn interacts with chromatin complexes, such as PRC2 (17) and Dnmt3a (23), breaking the symmetry between the future Xa (active X) and Xi. OCT4's developmental specificity thereby dictates timing of XCI (21, 29). In the same way, its developmental specificity influences other genetic targets, each of which initiates its own local and genome-wide cascades. The net effect of a single transcription factor on multiple downstream lncRNA targets would thus be multiplied by cycles of epigenetic reprogramming on gene-specific and network-wide scales, with each event relying on continuous collaborations among transcription factors, lncRNAs, and the epigenetic complexes recruited by the lncRNAs. The thousands of transcription factors and lncRNAs operating in parallel would then achieve the necessary local and genome-wide changes for development and for discrete responses to environmental signals.

Classes of lncRNAs

Genomic imprinting. Genomic imprinting is an epigenetic phenomenon in which genes are

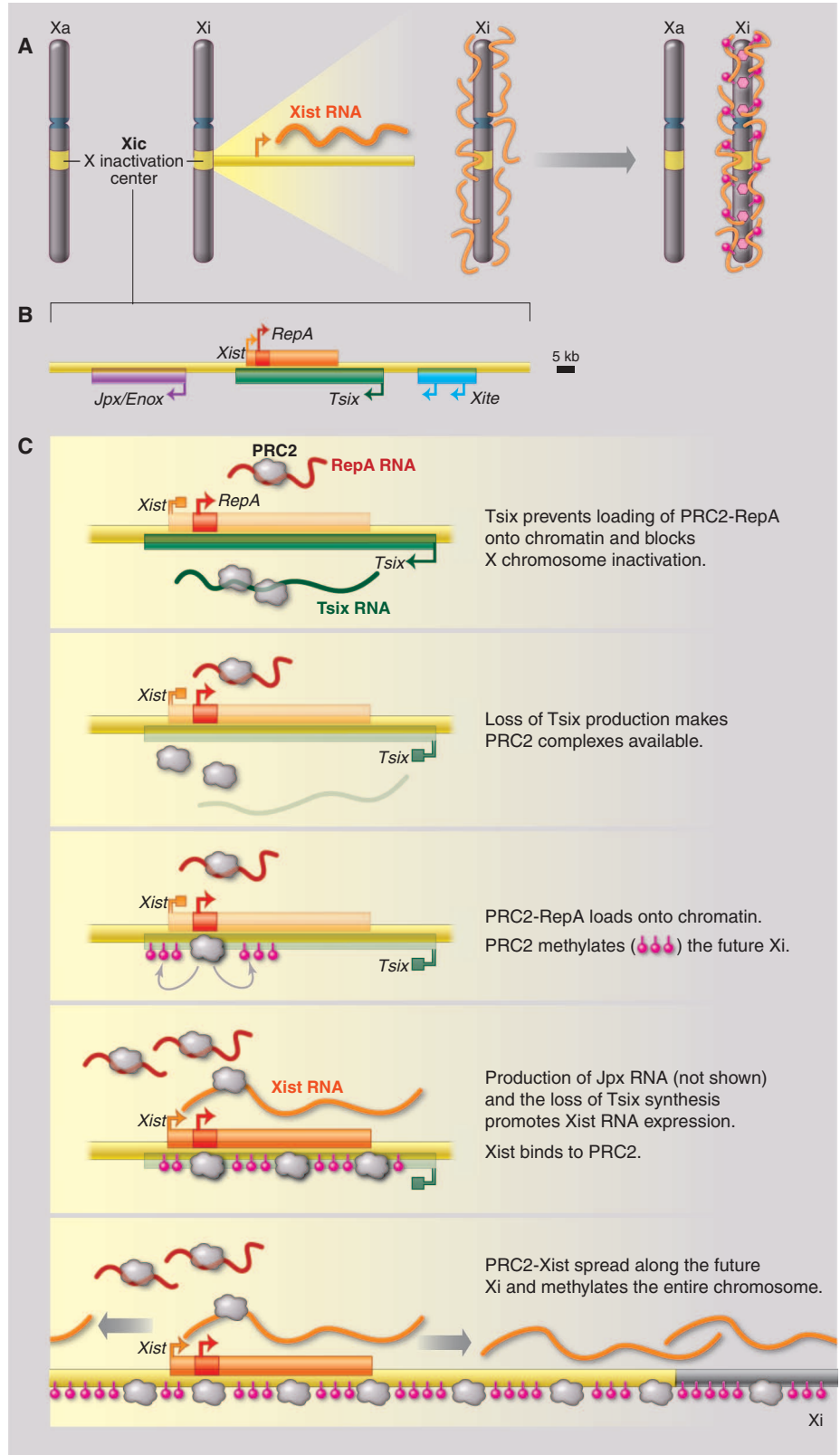


Fig. 1. lncRNAs in X-chromosome inactivation. **(A)** The lncRNA Xist is transcribed from the *Xic* of the inactive X chromosome (Xi). Xist RNA covers the entire chromosome and silences gene expression through epigenetic modification of histones and DNA. **(B)** The core region of the *Xic* and its lncRNAs. **(C)** lncRNA-protein interactions at the initiation of XCI.

expressed from the allele of only one parent (11, 12). The process bears a striking resemblance to XCI in that imprinting occurs within clusters and requires coordinated allelic regulation within the region, and its control regions are graced by lncRNAs. Whether these lncRNAs function as regulatory transcripts is currently debated. One of the first lncRNAs to be identified, H19 (30), is reciprocally imprinted with *insulin-like growth factor 2* (*Igf2*) and is highly expressed, but its

deletion has no phenotype (31). H19 now appears to function as microRNA (miRNA) precursor (32). In mice, the *Igf2* receptor gene (*Igf2r*) is regulated by the antisense *Airn* gene (33). Some genetic studies suggest that *Airn* regulates *Igf2r* through its act of transcription rather than through its lncRNA (34, 35). However, a biochemical study proposes that *Airn* RNA recruits the histone methyltransferase, G9a, to silence *Igf2r* (36). By using formaldehyde-cross-linked chromatin, the study left open the possibility that the contacts occur indirectly through a chromatin-bound intermediary rather than directly between lncRNA and G9a. Within the Beckwith-Wiedemann syndrome locus, a long antisense transcript (*Kncq1ot1*) may likewise associate with G9a and PRC2 (37). A genome-wide analysis using RNA immunoprecipitation-sequencing (RIP-seq) suggests that thousands of lncRNAs associate with PRC2 (38): At the *Dlk1-Gtl2* locus, *Gtl2* RNA may target PRC2 to the reciprocally imprinted *Dlk1* locus in cis; in the *Nesp/Gnas* cluster, the antisense transcript of *Nesp* (*Nespas*) may recruit PRC2 to control *Nesp* (38). Because the mechanisms of action remain to be investigated in detail, it is too soon to state whether these noncoding elements will have universal roles as lncRNAs in genomic imprinting.

Beyond allelic phenomena. lncRNA's function is not limited to the control of allelic expression (Table 1). In addition to the RIP-seq analysis identifying thousands of RNAs in the PRC2 transcriptome (38), analysis of RIP products on a microarray (RIP-chip) suggests that PRC2 and the LSD1/REST/coREST complex associate with hundreds of RNA (39–41) and that promoters of Polycomb target genes often make short transcripts that also associate with PRC2 (42). These PRC2 targets include hundreds that map to nonallelically regulated loci, including those involved in cancer and stem cell differentiation (38). The biochemically distinct Polycomb complex, PRC1, also interacts with RNA. The *INK4b/ARF/INK4a* tumor suppressor locus is controlled by PRC1 in a manner dependent on ANRIL lncRNA (43). Interestingly, interactions between a PRC1 subunit (PC2) and MALAT1 and TUG lncRNAs occur during movement of genes between nuclear compartments and during gene activation (44).

Noncoding RNAs frequently localize to gene promoters. These

promoter-associated short RNAs (PASRs) are typically short (50 to 200 nt) and have been considered abortive transcripts made by stalled or paused polymerases (42, 45–48). Pausing or stalling could prolong tethering and facilitate recruitment of factors (Fig. 2). For example, short lncRNAs made from *CCND1* (cyclin D1) may tether a transcription repressor, TLS, to the *CCND1* promoter and allosterically modify the repressor to turn off transcription (49). lncRNAs also occur in exons, introns, and other unusual spaces (8). In the plant *Arabidopsis thaliana*, the 1.1-kb intronic COLDAIR transcript controls flowering time by targeting PRC2 to silence *FLC* (*Flowering Locus C*) (50). In mice, RepA (within *Xist*'s first exon) targets PRC2 to the *Xist* promoter (17). Antisense transcripts may originate anywhere within or downstream of the genes they regulate [e.g., *Tsxi* and *Bdnf-as* (19, 51)]. lncRNAs also originate within retrotransposons, the ubiquitous repetitive elements once regarded as junk. Transcription from short interspersed element (SINE) B2, for example, may form a chromatin boundary for the growth hormone locus (52).

lncRNAs as activators. lncRNAs also serve as activators of gene expression. The brain-specific *Evl2* originates within an enhancer between *Dlx5* and *Dlx6* and is proposed to aid *Dlx2*-mediated activation of *Dlx5/Dlx6* (53). In XCI, *Jpx* RNA is required to induce *Xist* expression (25). From the *HoxA* cluster, two lncRNAs could recruit H3K4 trimethylases: Interaction between MLL and *Hottip* RNA is proposed to control activation of proximal *HoxA* genes (54), and interaction between MLL and *Mistral* RNA is thought to activate neighboring *HoxA6* and *HoxA7* genes (55). Recent studies showed abundant transcription through neuronal enhancers (56) and enhancer-like regions (6). Principles governing repressive lncRNA could also apply to activating lncRNAs, but much work remains to be done in this area because mechanistic details are currently lacking. In some cases, activation may depend on the act of transcription through associated chromatin rather than on the transcript.

Pseudogenes as regulators. Long regarded as a genomic graveyard, pseudogene space may turn out to be a vast repository of regulatory lncRNAs. For example, a pseudogene of *Makorin1* makes a truncated noncoding transcript that could stabilize the mRNA of the parent gene (57). The *asOct4-ps5* pseudogene could regulate *Oct4* by hybridizing with the *Oct4* mRNA, thereby targeting silencing complexes to the *Oct4* promoter (58). Pseudogenes of the tumor suppressor, *Pten*, have been proposed as decoys for miRNAs that bind to and down-regulate *Pten* mRNA (59). The proposed mechanisms remain controversial and require further investigation.

Fundamental Differences: Cis Versus Trans

lncRNAs operate not only in cis but also in trans (Table 1). Fundamental differences exist between these two categories. Cis-acting RNAs are restricted to the site of synthesis and directly act

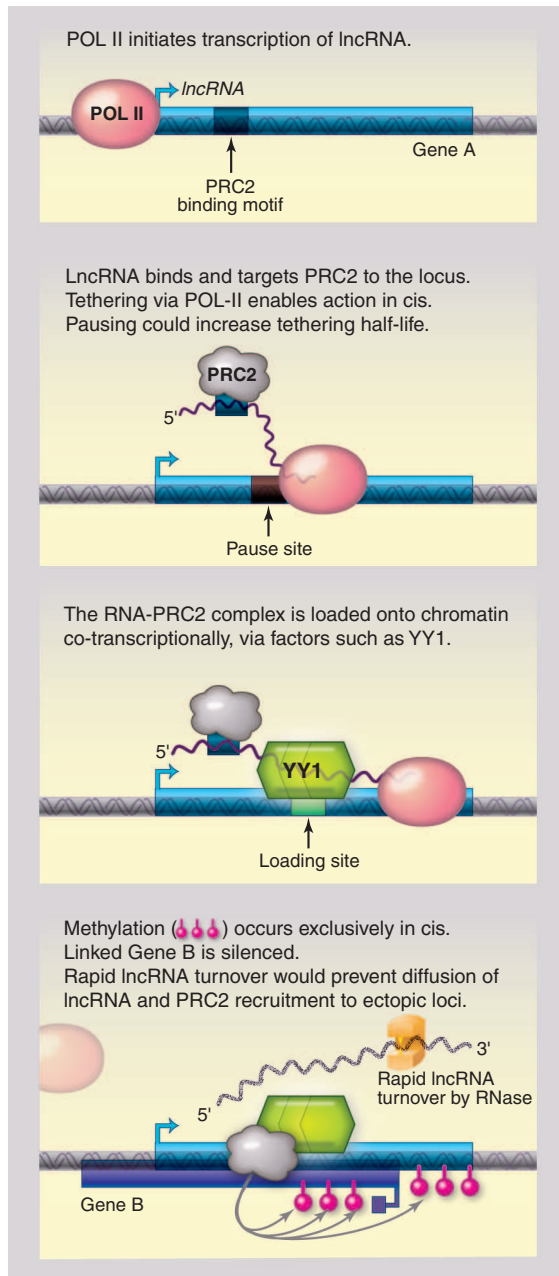


Fig. 2. lncRNAs tether epigenetic complexes to chromatin, enabling allele- and locus-specific regulation. lncRNA that is synthesized binds to an epigenetic complex (such as PRC2) and, together, are loaded onto chromatin cotranscriptionally through DNA-bound factors (such as YY1 for *Xist* RNA). Epigenetic modifications then silence the gene, and rapid lncRNA turnover prevents its diffusion to other loci.

on one or several linked, generally contiguous, genes on the same chromosome. By contrast, trans-acting RNAs diffuse from the site of synthesis and can act directly on many genes at great distances, including at other chromosomes. In doing so, trans-acting RNAs, like transcription factors and small RNAs, are more likely to converge on and act within large genic networks. Both mechanisms imply direct action on target genes rather than secondary downstream effects. Whereas cis-acting lncRNAs are exemplified by mammalian XCI, trans-acting lncRNAs are exemplified by fruit fly dosage compensation, which occurs by hypertranscribing the single male X chromosome and which therefore does not require allelic discrimination. Indeed, the lncRNAs, roX1 and roX2 (60, 61), scaffold the MSL-MOF protein complex, which in turn binds hundreds of X-linked sites (62, 63).

Whether mammalian lncRNAs generally work in cis or trans has been debated (6, 7, 40, 56, 64, 65). There will likely be many members in each class. Some might even blur the distinction. Xist RNA, for example, acts in cis to repress genes on Xi in the normal context but could diffuse to ectopic sites when *Xist* transgenes are introduced de novo (18). This observation indicates that *Xist* is actually diffusible but is prevented from acting in trans by

developmental programming that masks non-*Xic* YY1-binding sites. Thus, cis-acting transcripts may operate in cis only to the extent that they are properly anchored and ectopic sites masked.

Although mechanistically and consequentially very different, the *Xist* case illustrates why cis versus trans lncRNAs are not always easily distinguished. Complicating the matter is a lack of knowledge of how the vast majority of lncRNAs work. One key unanswered question is whether trans-acting lncRNAs could also target chromatin complexes. The property of targeting is reserved for cases in which RNA is directly involved in guiding a complex to a specific locus. Targeting is easier to conceive for cis-regulatory RNAs, which are naturally tethered to the site of transcription (Fig. 2). A targeting role for diffusible, trans-acting RNA is possible, however, if they engage in RNA:DNA triplex formation via Hoogsteen base-pairing, as has been proposed for pRNA (promoter-associated)-mediated recruitment of DNMT3b to ribosomal gene (rDNA) promoters (66). Another hypothesized targeting mechanism involves hybridization between complementary RNA strands of the antisense *Oct4* pseudogene and the target *Oct4* mRNA (58).

Site-specific targeting seems unlikely to be a property of most trans-acting lncRNAs. Existing

examples suggest that many have functions akin to those of protein factors. Steroid receptor RNA (SRA), a “subunit” of nuclear hormone receptors for estrogen, androgen, glucocorticoid, and progesterone (67), functions as a coactivator in the same way as traditional protein-based coactivators such as p300 and the transcription factor cyclic adenosine monophosphate (cAMP) response element binding protein (CREB). SINE B2 RNAs, a product of retrotransposons, directly bind Pol II and down-regulate Pol II activity at mammalian heat-shock genes, thereby functioning like protein-based co-repressors (68). lncRNAs also scaffold protein complexes. HOTAIR from the *HOX-C* locus (39) scaffolds PRC2 and LSD1 (a histone H3K4 demethylase) (41, 69), and genome-wide analyses show that it localizes to thousands of sites in disease states (62, 70) as well as to the *HOX-D* cluster in trans (39). Other functions of trans-acting lncRNAs are only beginning to emerge, and many remain incompletely defined. The expression of an lncRNA called lincRNA-p21 and the p21-associated ncRNA DNA damage-activated (PANDA) lncRNA (71, 72) are induced during the p53 response and correlate with gene expression changes. The abundant MALAT1 and TUG1 lncRNAs differentially interact with methylated and unmethylated forms of PC2 (PRC1 subunit) to relocate growth-control genes between nuclear substructures for transcription activation (44). Thus, unlike the site-specific cis-acting lncRNAs, trans-acting transcripts operate within large genic networks.

Table 1. Emerging themes in lncRNA regulation. Potential groupings of lncRNA based on proposed interactions, functions, and mechanisms. Representative lncRNAs of each group are shown.

Functions	Examples	Action	Hypothesized mechanisms
Cis-tether cis-targeting	RepA, <i>Xist</i> , <i>Tsix</i> , <i>Gtl2</i> , <i>Kcnq1ot1</i> , <i>Airn</i> , <i>Hottip</i> , <i>ANRIL</i> , <i>Oct4-ps5</i> , <i>COLDAIR</i> , <i>Evf2</i> , <i>BDNF-AS</i>	Cis	Co-transcriptional targeting of chromatin factors; allelic and locus- specific action; repressive or activating
Trans-targeting	pRNA, <i>asOct4-ps5</i>	Trans	RNA:DNA triplex via Hoogsteen base pairing or sense:antisense Watson:Crick base pairing
Enhancer	<i>Xite</i> , ncRNA-a7, eRNAs	Cis	Mediated by RNA, transcriptional force, or chromatin intermediaries
Decoy	<i>PTEN-ps</i> <i>Tsix</i>	Trans Cis	Competitive inhibition of target protein or RNA
Scaffold	<i>MALAT1</i> , <i>TUG1</i> , <i>NEAT1</i> , <i>HOTAIR</i> , <i>roX1</i> , <i>roX2</i>	Trans	RNA subunit of effector complex
Allosteric modification	<i>TLS</i>	Cis (trans also possible)	Alters conformation and activity of protein partners
Coactivator or co-repressor	<i>SRA</i> , <i>SINE B2</i> , <i>Jpx</i> , pRNA	Trans (cis also possible)	Accessory factor for transcriptional activation or repression

Prospects and Conclusions

The lncRNA field, once a boutique field for imprinting and dosage compensation, has now grown to include mainstream biochemists, genomicists, and computational biologists. The accelerated discovery of thousands of lncRNAs over the past 5 years is outpacing our ability to vet function and mechanism. Indeed, few lncRNA knockouts have yielded robust phenotypes. *Hi9* knockout mice have a normal phenotype (31). A *HoxC* deletion including *Hotair* does not abolish PRC2 targeting (73). Knockouts of *Malat1* and *Neat1*, two of the most abundant nuclear lncRNAs, yielded normal, viable mice (74–76). Although these findings lead some to question the importance other lncRNAs, they do not exclude the possibility of developmental compensation, as often happens in knockout models, nor do they exclude the possibility that subtle effects would emerge in tissue-specific studies.

Related issues are the inherent differences in comparing knockout to knockdown phenotypes. Before RNAi, gene deletions were the staple of proving function in vivo. Reduction of gene expression by small RNAs may not recapitulate knockouts, especially because nuclear lncRNAs are often difficult to knock down and residual levels of lncRNAs after a knockdown could yield phenotypes different from knockouts that completely eliminate lncRNA production. Analysis of lncRNA function would ideally also include

gain-of-function experiments, because exaggerated phenotypes would bolster arguments for proposed effects.

Equally important considerations include experiments to distinguish among RNA, transcriptional activity, and underlying chromatin as the mechanism of action for a noncoding locus. Because they are inextricably linked, distinguishing among them may not be trivial. The movement of Pol II unwinds the DNA duplex, alters chromatin structure, and could effect epigenetic change independently of the nascent transcript. For example, transcript truncation experiments have shown that the lncRNA made from *Tsix*'s enhancer, *Xite*, does not equate with deleting *Xite*, suggesting that *Xite* operates through its transcription or chromatin change rather than through *Xite* RNA (77). A similar conclusion was reached regarding transcription of *Airn* (35). Thus, transcript truncation experiments might be generally helpful in analysis of lncRNA function. When the RNA per se is shown to be necessary, what they recruit and how they interact with protein partners would need elucidation before general principles could be articulated. To date, few mammalian lncRNAs have been tested in these ways.

Examples discussed herein caution against treating all lncRNAs as a single class of molecules. Some classification schemes have been based on geography, resulting in the coinage of catchy names like PASR for promoter-associated short RNAs (45), NAT for natural antisense transcripts (78), lincRNA for large intervening noncoding RNA (2), and eRNA for enhancer-associated RNAs (6, 56). Yet a transcript's location may not instruct function or mechanism. *Jpx* resides in cis to *Xist*, but *Jpx* acts in trans (25), and an antisense orientation does not necessarily imply repressive effects (6). Distinction by cis versus trans action might be a logical first step. Eventually, it will be useful to think in terms of transcriptomes. Although a transcriptome has so far only been identified for PRC2 (17, 38–40, 42), those for other epigenetic complexes would not be difficult to envision.

The mechanisms outlined in Table 1 likely provide only a glimpse of the full range of lncRNA functions as others await discovery. In addition to the more traditional roles in scaffolding, coactivation, and co-repression of trans-acting RNAs, cis-acting lncRNAs are now known to perform crucial targeting functions in a site-specific manner. Until recently, the proteome was thought to possess its own targeting specificity. lncRNAs are now known to confer a degree of temporal and spatial specificity not possible with proteins and small RNAs. Allelic and locus-

specific control might have been a major driving force behind the genome-wide fixation of lncRNAs. Eventually, lncRNAs may be seen to play roles as far-reaching as small RNAs and proteins, given that disease-associated mutations occur more often in coding than noncoding space (79) and that species-specific differences among organisms now appear to originate more in noncoding space than in the few thousand coding genes we share. With the site-specific action of cis-acting lncRNAs, drugs designed against lncRNAs could circumvent pleiotropic effects that plague many current treatment modalities that target enzymatic activities within epigenetic complexes. Indeed, the Wild West is a rich landscape waiting to unfold.

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Aggravating Genetic Interactions Allow a Solution to Redundancy in a Bacterial Pathogen

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Interactions between hosts and pathogens are complex, so understanding the events that govern these interactions requires the analysis of molecular mechanisms operating in both organisms. Many pathogens use multiple strategies to target a single event in the disease process, confounding the identification of the important determinants of virulence. We developed a genetic screening strategy called insertional mutagenesis and depletion (iMAD) that combines bacterial mutagenesis and RNA interference, to systematically dissect the interplay between a pathogen and its host. We used this technique to resolve the network of proteins secreted by the bacterium *Legionella pneumophila* to promote intracellular growth, a critical determinant of pathogenicity of this organism. This strategy is broadly applicable, allowing the dissection of any interface between two organisms involving numerous interactions.

The outcome of most parasitic relationships is decided by a series of molecular interactions involving hundreds of proteins. Through specialized secretion systems, intracellular pathogens deploy an arsenal of proteins that modulate numerous host cell processes to establish growth (1). For many pathogens, redundancy complicates the analysis of these secreted virulence proteins (2), and no systematic strategy exists that allows specific roles to be ascribed to proteins that, on the basis of mutant analysis, appear to be dispensable for growth within the host.

Legionella pneumophila is a parasite of a broad range of free-living amoebae (3). Human disease occurs after inhalation of contaminated water aerosols followed by replication of the bacteria in alveolar macrophages (4), which results in pneumonia. Within the host cell, *L. pneumophila* establishes replication within a membrane-bound compartment by preventing the delivery of this vacuole to the lysosome (5), while recruiting host material from the endoplasmic reticulum (ER) (6). This latter event is accomplished by manipulating host proteins involved in the early secretory pathway (7), including the small guanosine triphosphatases (GTPases) Rab1, Arf1, and Sar1 as well as Sec22 and Bet5, proteins involved in tethering and fusion of ER-derived vesicles to target membranes (7–9).

Intracellular replication of *L. pneumophila* depends on the Dot/Icm type IVb secretion system (10, 11). To date, more than 270 *L. pneumophila* proteins have been shown to be substrates of

Dot/Icm (12), several of which directly modulate the activity of GTPases that control host vesicle trafficking. Although the biochemical activity of more than a dozen Dot/Icm translocated substrates (TSs) and their host targets are known, their roles in disease are unclear, because deletion of their encoding genes rarely causes a detectable defect in intracellular growth (13). The most likely explanation for this is redundancy, which extends beyond multiple paralogs that can perform the same function (13, 14) to the use of seemingly unrelated proteins to manipulate complementary host cell pathways (9). Although this has impeded understanding how these proteins contribute to pathogenesis, there has been no attempt to systematically resolve this problem.

iMAD defines host-bacterial gene pairs important for intracellular growth. Studies using RNA interference (RNAi) in cultured *Drosophila* cells demonstrate that wild-type *L. pneumophila* grow efficiently in cells depleted of one of the membrane trafficking proteins Arf1, Sec22, or Bet5; however, simultaneous depletion of pairs of these proteins impairs intracellular replication of the bacterium (9). These data are consistent with several different pathways promoting intracellular replication, with bacterial growth inhibition requiring disruption of at least two pathways. We reasoned that mutation of a bacterial gene encoding a Dot/Icm TS that targets a host pathway distinct from one targeted by RNAi should effectively abolish the contribution of both pathways (fig. S1).

Using transposon site hybridization (TraSH) (15), we screened a library of *L. pneumophila* transposon mutants (16) for mutations that render the bacterium defective for growth in *Drosophila* cells depleted of various component proteins of the early secretory system (fig. S1). A total of 678 genes were identified as being important for intracellular growth in *Drosophila* cells in the pres-

ence of double-stranded RNA (dsRNA) treatment (table S1). Many of these encode proteins that are not predicted to function in the host cytoplasm. For example, several genes encode proteins involved in nutrient acquisition and metabolism (table S1), consistent with disruptions in vacuole remodeling limiting the availability of metabolites required for growth. To address the inability to connect phenotypes with mutations in Dot/Icm TS genes, one of the central problems in the field, we focused on the 55 Dot/Icm TS-encoding genes identified (Fig. 1A), 44 of which had not previously been shown to have a role in promoting intracellular growth of *L. pneumophila*.

Data from the iMAD screen could be recapitulated for selected genes when *Drosophila* cells treated with the appropriate dsRNA were challenged with *L. pneumophila* deletion mutants (Fig. 1B). For example, a *L. pneumophila* $\Delta wipB$ (*lpg0642*) mutant was defective for growth in cells depleted of Sec22 but not in untreated *Drosophila* cells or in cells depleted of Bet5, as predicted from our screen. Similarly, growth of a $\Delta ceg32/sidI$ (*lpg2504*) mutant was more defective in cells depleted of Arf1 than in untreated cells or cells depleted of Sar1 (Fig. 1B). Thus, we have mapped a series of aggravating genetic interactions (17) that identify specific host factors required for intracellular replication of bacterial mutants. These results defined host conditions under which individual bacterial proteins were important for growth and provided the basis for identifying Dot/Icm TSs that function in complementary host pathways.

Identification of redundant relationships between Dot/Icm translocated substrates. We predicted that if a set of Dot/Icm TSs targets a particular host pathway, mutations in individual members of that set should result in similar phenotypes. Our iMAD screen examined growth of *L. pneumophila* mutants in *Drosophila* cells under five different conditions of dsRNA treatment. Using these data, we performed cluster analysis to group bacterial mutations on the basis of common behavior patterns. This identified 14 distinct functional groups of Dot/Icm TSs with more than one member (Fig. 1A; groups I to XIV). For example, mutations in the *L. pneumophila* gene *lidA* impaired intracellular growth if either Bet5 or Sec22 was depleted, and mutations in *legA3* (*lpg2300*) behaved similarly, placing *legA3* and *lidA* in the same functional group (Fig. 1A; group XI). Conversely, mutation of *wipB* caused defective intracellular growth in cells depleted of either Arf1 or Sec22, placing it in a separate functional group (Fig. 1A; group IX). If two functional groups consist of bacterial proteins that modulate redundant host pathways, we predicted that the combined deletion of one bacterial gene from each functional group should result in defective intracellular replication of *L. pneumophila* in untreated *Drosophila* cells. LidA and WipB clustered to separate functional groups, predicting that they act on potentially redundant host cell pathways,

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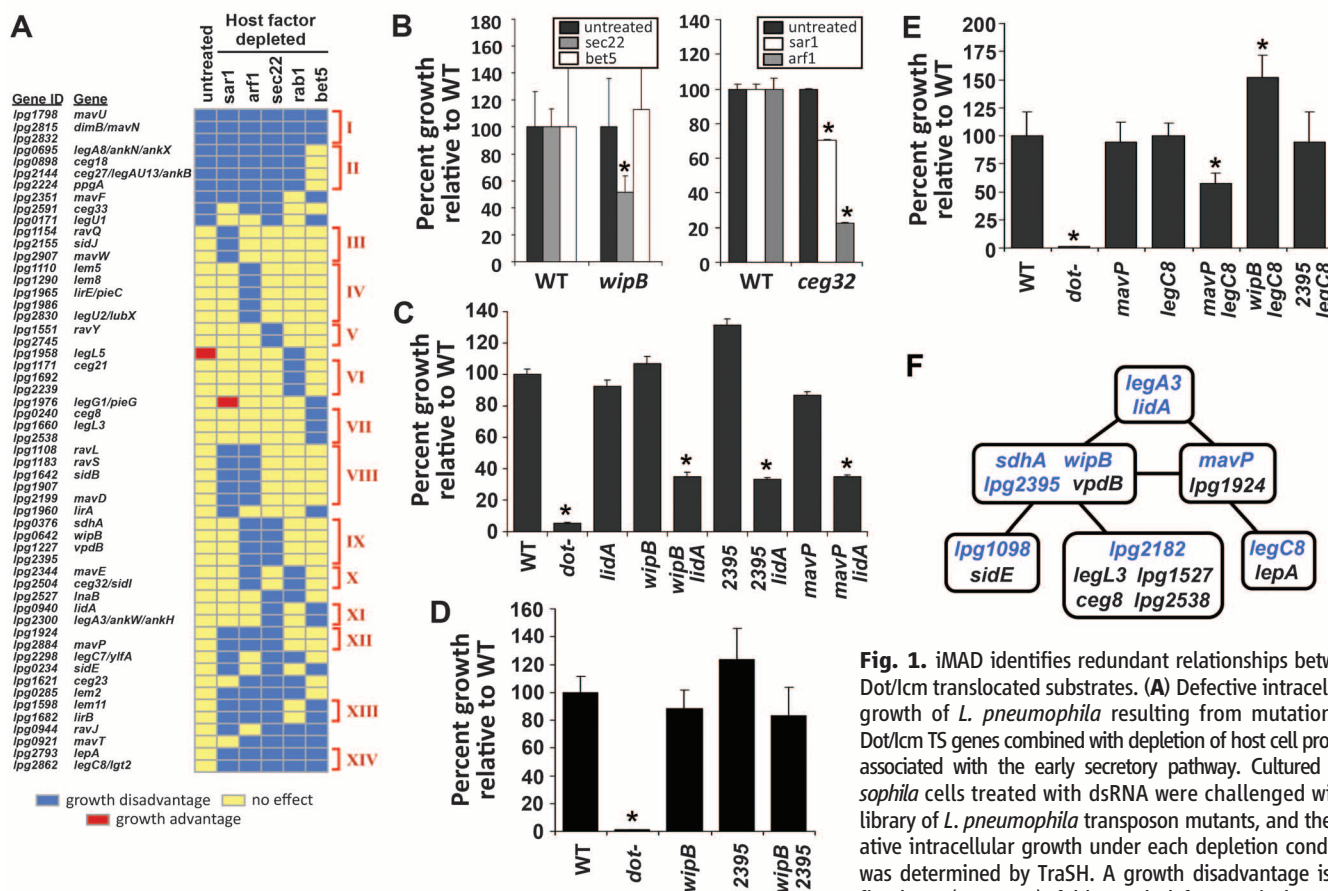
so growth of a $\Delta wipB\Delta lidA$ double mutant was assessed in untreated *Drosophila* cells. Consistent with the interpretation that these two proteins target redundant pathways, the double mutant showed reduced intracellular replication in untreated *Drosophila* cells compared to either wild-type *L. pneumophila* or strains bearing mutations in only one of these genes (Fig. 1C). Mutations in a newly identified Dot/Icm TS-encoding gene, *lpg2395* (fig. S2), clustered to the same functional group as *wipB* mutations (Fig. 1A: group IX), and as predicted by this strategy, deletion of *lpg2395* impaired growth of *L. pneumophila* when combined with $\Delta lidA$ (Fig. 1C). By contrast, a strain containing lesions in two genes assigned to the same functional group, $\Delta wipB\Delta lpg2395$, grew as proficiently as the wild-type strain in untreated *Drosophila* cells, consistent with the model that growth defects are only observed when target-

ing of multiple host pathways by Dot/Icm TS is disrupted (Fig. 1D). This demonstrated that cluster analysis can predict pairs of seemingly unrelated Dot/Icm TSs that perform compensatory functions.

Defective intracellular growth was not limited to strains that combine mutations from functional groups IX and XI. Mutations in *mavP* (*lpg2884*) clustered in a third functional group, group XII (Fig. 1A), and the combined deletion of *mavP* with *lidA* similarly impaired bacterial growth in untreated *Drosophila* cells (Fig. 1C). We also observed specificity between functional groups. For example, reduced growth of *L. pneumophila* was observed when *legC8* (*lgt2/lpg2862*) (group XIV) was deleted in combination with *mavP* (Fig. 1E). However, neither $\Delta legC8\Delta wipB$ nor $\Delta legC8\Delta lpg2395$ double mutants were attenuated, despite loss of gene pairs from separate func-

tional groups (Fig. 1E). Instead, we observed a positive interaction between mutations in *wipB* and *legC8* in which intracellular growth was enhanced by combining the two mutations (Fig. 1E). Thus, cluster analysis of mutations allows functional relationships between bacterial proteins to be revealed.

By analyzing genetic interactions between members of several functional groups, we could identify a network of functional relationships between *L. pneumophila* proteins, in which pairs of mutations in Dot/Icm TS genes were predicted to have aggravating effects on intracellular growth (Fig. 1F, lines represent predicted aggravating genetic interactions). Many of these predicted interactions were verified by constructing defined double deletions (Fig. 1F, blue font). The cluster analysis also assigned genes encoding proteins of unknown function, such as *lpg2182* and *lpg1098*



average TraSH ratio $>1.75 \pm 0.1$ SDs from the mean, which varies depending on the dsRNA treatment. Individual genes are clustered into distinct functional groups (red brackets, I to XIV) on the basis of common behavioral patterns across all host conditions examined. (B) Host condition-specific growth defects for *L. pneumophila* null mutants. Growth of *wipB* (*lpg0642*) (group IX) (left) and *ceg32/sidI* (*lpg2504*) (group X) (right) null mutants in cultured *Drosophila* cells depleted of the indicated proteins by RNAi was compared to that of the wild-type (WT) strain. (C) The combined deletion of bacterial genes from separate functional groups impaired intracellular replication of *L. pneumophila* in untreated *Drosophila* cells. (D) Deletion of genes belonging to the same functional group did not adversely affect bacterial growth in untreated *Drosophila* cells. (E) Genetic interactions define distinct relationships between different functional groups. (F) Summary of redundant relationships between individual functional groups defined by genetic interaction mapping. A solid black line indicates aggravating genetic interactions on intracellular growth of the bacterium in untreated *Drosophila* cells when two *L. pneumophila* genes, one from each of the corresponding functional groups (indicated by blue lettering), are deleted in combination. (B to E) Bacterial growth was determined by colony-forming units (CFU) on solid media from lysed host cells 48 hours after infection relative to the number of CFU recovered 1 hour after infection. Data are means from at least two independent experiments $\pm$ SD of three replicates. $*P < 0.05$ relative to the WT strain.

(table S1), to specific functional groups (Fig. 1F), allowing these proteins to be assigned to particular steps in intracellular growth, defined by the members of their respective functional groups.

Redundant Dot/Icm translocated substrate-targeted host pathways contribute to vacuole biogenesis in natural hosts. To show that the functional relationships between Dot/Icm TSSs identified with iMAD were similarly important in a natural host of *L. pneumophila*, we examined the growth of double mutants in *Acanthamoeba castellanii*. Each of the double mutants shown to be defective for replication in *Drosophila* cells exhibited impaired intracellular replication in the amoebal host (Fig. 2A and fig. S3). Growth of all the double mutants could be rescued by reintroducing either of the deleted genes individually, and no growth defects were observed for any of the double mutants when grown in bacteriological medium (fig. S3). A more detailed examination through a single round of infection in *A. castellanii* revealed that all three double mutants generated vacuoles that contained fewer bacteria than the wild-type strain (Fig. 2B and fig. S3). This phenotype correlated with inefficient recruitment of ER-derived material, as determined in *Dictyostelium discoideum* by measuring accumulation of the GFP-HDEL protein about the *Legionella*-containing vacuole (LCV). GFP-HDEL is a fusion protein of the green fluorescent protein (GFP) and the ER localization signal HDEL, which is used as a proxy for the presence of ER-resident proteins in amoebae (5, 18, 19). In *D. discoideum* harboring GFP-HDEL, fewer $\Delta wipB\Delta lidA$ -containing vacuoles stained positively for this marker compared to amoebae challenged with either the wild-type strain or bacteria containing a single mutation (Fig. 2C), supporting the model that the activities of WipB and LidA can compensate for each other's absence. As expected, the $\Delta wipB\Delta lidA$ mutant grew less robustly than either the wild-type or single-deletion strains in this host (Fig. 2D). These results are consistent with at least two routes for generating a replication compartment that are targeted by *L. pneumophila* proteins in all the host cells examined. The partial defect in GFP-HDEL recruitment and intracellular growth of the $\Delta wipB\Delta lidA$ mutant likely reflects the existence of additional pathways that can partially compensate for loss of WipB and LidA.

Hierarchical clustering predicts phenotypic defects resulting from loss of bacterial proteins. Mutations placed in the same group by hierarchical cluster analysis identify proteins that participate in the same process in the cell or that constitute a single protein complex (20). We applied the same reasoning to the cluster analysis data obtained from our iMAD screen in *Drosophila* cells, which indicated that mutations in *wipB* and *lpg2395* belonged to the same functional group as *sdhA* mutations (Fig. 1A), consistent with all three proteins contributing to a common step in LCV formation. SdhA plays a crucial role in maintaining LCV integrity in bone marrow-derived murine macrophages (21). Vacuoles harboring $\Delta sdhA$

mutant bacteria are more permeable than those harboring wild-type bacteria (21), and as a consequence colocalize with host proteins associated with partially degraded phagosomes (21, 22). This vacuolar disruption leads to host cell death, bacterial degradation, and poor bacterial growth in macrophages challenged with $\Delta sdhA$ mutants (21, 23). Therefore, we tested whether mutations that cluster with *sdhA* lesions could identify other *L. pneumophila* proteins that promote vacuole integrity. Consistent with other cell types, the $\Delta wipB\Delta lidA$ double mutant exhibited reduced replication in bone marrow-derived murine macrophages relative to either the wild-type strain or strains having single mutations in each of these

genes (Fig. 3A). Because a *L. pneumophila* mutant lacking 31% of Dot/Icm TSSs grows robustly in macrophages (16), this finding confirmed the power of the cluster analysis to identify functionally related Dot/Icm TSSs. The growth defect was associated with a loss of membrane integrity of the LCV on the basis of colocalization with Galectin-3, a marker for vacuole lysis (22). Macrophages challenged with the $\Delta wipB\Delta lidA$ mutant showed an increase relative to wild-type bacteria in the number of vacuoles staining positively for Galectin-3 (Fig. 3B). Localization of this marker was comparable to challenge with the $\Delta sdhA$ mutant, whereas the $\Delta wipB$ and $\Delta lidA$ single-deletion mutants resembled the wild-type strain in this assay (Fig. 3B).

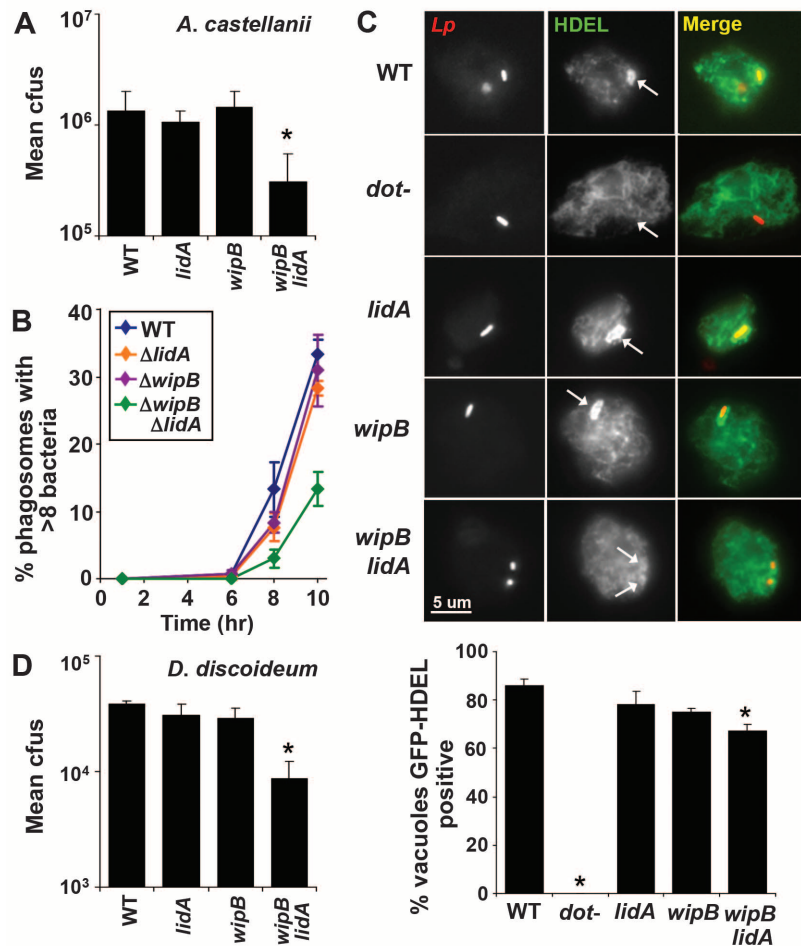


Fig. 2. Impaired growth of *L. pneumophila* mutants in natural hosts. (A) The $\Delta wipB\Delta lidA$ mutant shows a growth defect in *A. castellanii* relative to the WT and corresponding single-deletion strains. (B) The $\Delta wipB\Delta lidA$ mutant is impaired in the accumulation of vacuoles containing large numbers of bacteria. *A. castellanii* were infected with *L. pneumophila* strains expressing GFP, then fixed 1, 6, 8, or 10 hours after infection; the number of bacteria per phagosome was scored by means of fluorescence microscopy. (C) The $\Delta wipB\Delta lidA$ mutant is defective for recruitment of the ER-targeted fusion protein GFP-HDEL in *D. discoideum*. *D. discoideum* expressing GFP-HDEL were infected with *L. pneumophila* strains expressing the red fluorescent protein tdTomato for 4 hours, fixed, and visualized by fluorescence microscopy (upper panel). The number of GFP-HDEL-positive *Legionella* vacuoles was scored, counting 100 vacuoles per replicate (lower panel). (D) Defective intracellular growth of the $\Delta wipB\Delta lidA$ mutant in *D. discoideum*. (A and D) *A. castellanii* and *D. discoideum* were infected with *L. pneumophila* and bacterial growth was monitored as described in Fig. 1 over 3 days, equivalent to three consecutive rounds of replication and plotted as in Fig. 1. Data are representative of at least two independent experiments $\pm$ SD of three replicates. * $P < 0.05$ relative to the WT strain.

Analysis of morphological markers was consistent with the $\Delta wipB\Delta lidA$ strain causing a breakdown in vacuole integrity (21). We observed an increase in the number of infected host cells undergoing cell death relative to cells harboring

wild-type bacteria (Fig. 3C), indicated by aberrant nuclear morphology characteristic of apoptosis, and the number of bacteria appearing degraded, on the basis of aberrant bacterial morphology (Fig. 3D), was similarly increased. The amount of bac-

terial degradation and host cell death was less extensive than that observed for the $\Delta sdhA$ mutant, consistent with the relative severity of the growth defects of these two strains. For the $\Delta sdhA$ mutant, the vast majority of vacuoles contained a single bacterium, whereas vacuoles harboring the $\Delta wipB\Delta lidA$ mutant often had more than one bacterium, many of which appeared degraded (Fig. 3D). This is consistent with failure of the double mutant to maintain vacuole integrity, even though there was sufficient vacuolar remodeling to support initial replication events (Fig. 3, D and E). Enhanced recruitment of Galectin-3 and host cell death relative to the wild-type strain was similarly observed in macrophages challenged with the $\Delta mavP\Delta lidA$ mutant, accompanying defective growth of this mutant in macrophages (fig. S4). Assigning genes to specific functional groups based on the behavior of their corresponding mutants, therefore, identified microbial proteins that were required for similar steps during replication vacuole biogenesis.

Host-specific requirements for pairs of Dot/Icm translocated substrates. Despite its severe growth defect in macrophages, an *L. pneumophila* $\Delta sdhA$ mutant is only partially attenuated for growth in *D. discoideum*, presumably because the host cell death pathways that inhibit growth of this mutant in macrophages are not present in amoebae (23). The cluster analysis predicted that this partial defect should be potentiated by the addition of a second mutation known to aggravate mutations that belong to the same functional group as *sdhA* (group IX) (Fig. 1A). As mutations in group IX (*wipB* and *lpg2395*) were aggravated by mutations in group XI (*lidA* and *legA3*) (Fig. 2, B and D), we predicted that a group XI mutation, such as deletion of *lidA*, should exacerbate the growth defect of the $\Delta sdhA$ mutant in *D. discoideum*. Indeed, the $\Delta sdhA\Delta lidA$ double mutant exhibited reduced growth relative to the $\Delta sdhA$ mutant alone (Fig. 4A). Moreover, a mutant lacking *sdhA* and *legA3*, another member of group XI, was also attenuated for growth in this host (Fig. 4A). Thus, the presence of group XI proteins could compensate for loss of SdhA in this amoebal host.

Phenotypic analysis of *L. pneumophila* double mutants showed that group XI in its entirety was required to bypass the loss of single members of group IX; however, the extent to which individual members were able to compensate varied. For example, in *A. castellanii*, the $\Delta sdhA\Delta lidA$ mutant was more attenuated for growth than the $\Delta sdhA\Delta legA3$ mutant (Fig. 4B), suggesting a more substantial role for LidA than LegA3 in promoting growth in the absence of SdhA in this species. This phenomenon was dependent on the host examined, as we did not observe this hierarchical relationship in *D. discoideum* (Fig. 4A). Similarly, in bone marrow–derived macrophages, loss of *mavP* had no effect on the growth of a $\Delta legA3$ mutant (Fig. 4C), whereas the $\Delta mavP\Delta legA3$ mutant showed reduced growth in *A. castellanii* (Fig. 4D). Thus, optimal growth in a particular host depends on a

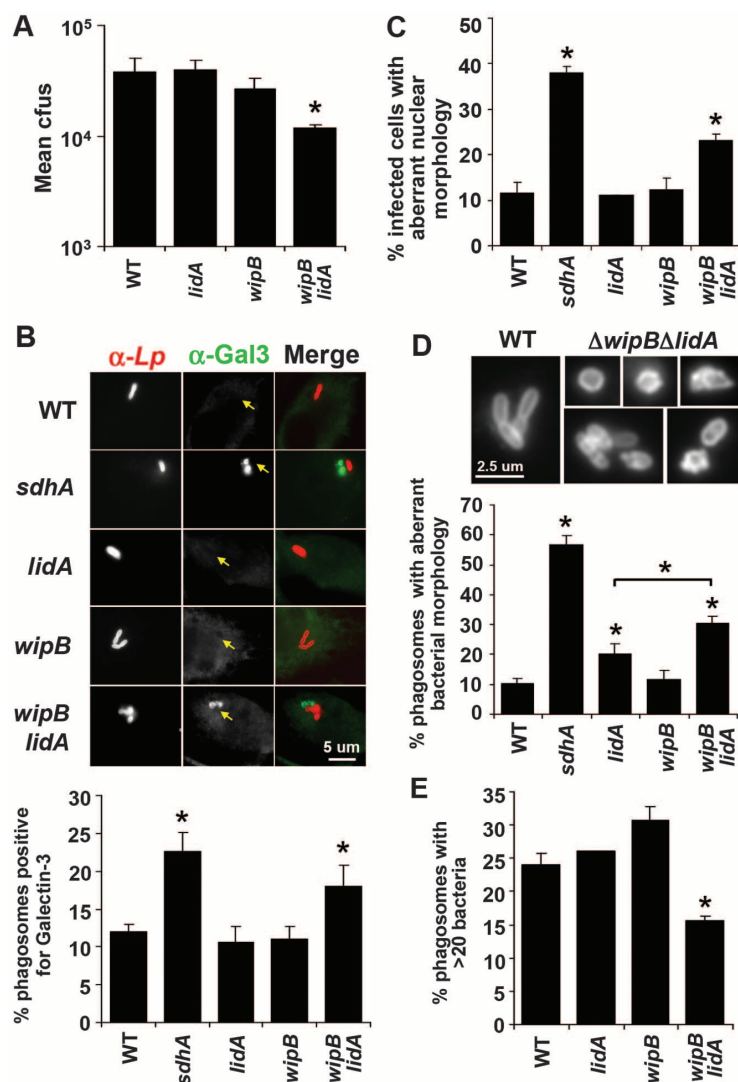


Fig. 3. Hierarchical cluster analysis predicts double mutants defective in maintaining vacuole integrity. (A) Growth of the $\Delta wipB\Delta lidA$ mutant was reduced in bone marrow–derived murine macrophages compared to the WT and single-deletion mutant strains. Bacterial growth was determined as described in Fig. 2. (B) $\Delta wipB\Delta lidA$ mutant-containing vacuoles showed enhanced recruitment of Galectin-3. Macrophages were infected with *L. pneumophila* for 6 hours, fixed and stained for *Legionella* and Galectin-3, then visualized by fluorescence microscopy (upper panel). The number of *Legionella* vacuoles staining positive for Galectin-3 was scored (lower panel). (C) Cells infected with the $\Delta wipB\Delta lidA$ mutant exhibit increased host cell death based on aberrant nuclear morphology characteristic of apoptosis. Macrophages were infected with *L. pneumophila* for 8 hours, fixed and stained for *Legionella*, then treated with Hoechst stain. (D) $\Delta wipB\Delta lidA$ mutant bacteria exhibited aberrant morphology after challenge of macrophages. Bacteria visualized by fluorescence microscopy as in (B) showed both swelling and blebbing in vacuoles containing either single or multiple bacteria relative to the smooth rod-shaped morphology of WT bacteria (upper panel). The number of vacuoles in which at least one bacterium exhibited aberrant morphology was scored (bottom panel). (E) The $\Delta wipB\Delta lidA$ mutant showed a defect in the number of vacuoles containing large numbers of bacteria in a macrophage host. Macrophages were infected with *L. pneumophila* for 10 hours, fixed, and stained for *Legionella*, and the number of bacteria per vacuole was scored. (A to E) Data are representative of at least two independent experiments $\pm$ SD of three replicates, scoring 100 vacuoles (B, D, and E) or infected cells (C) per replicate. * $P < 0.05$ relative to the WT strain unless indicated otherwise.

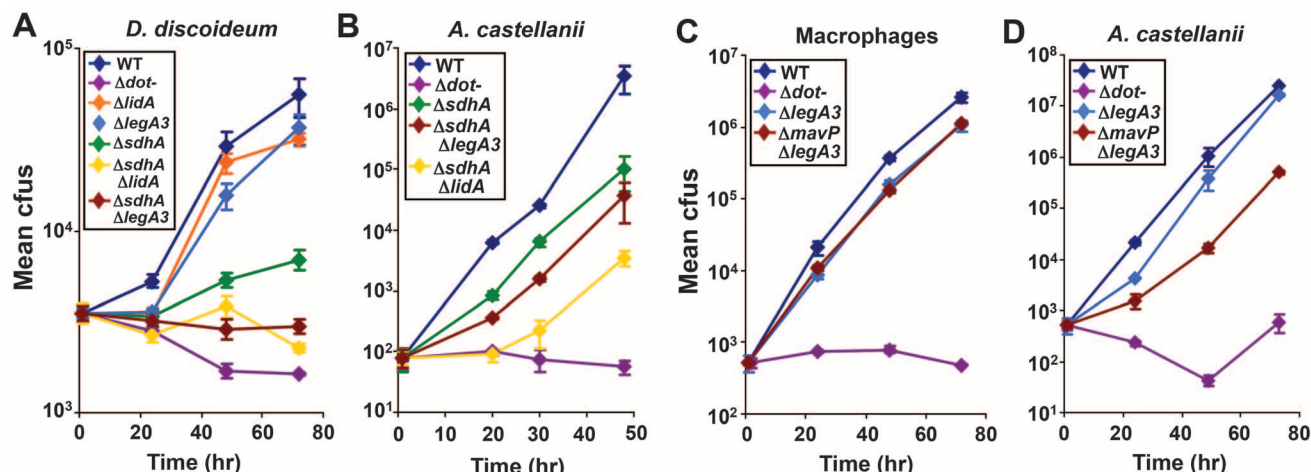


Fig. 4. Host-specific aggravating genetic interactions between mutations in genes encoding Dot/Icm translocated substrates. (A) Deletion of *lidA* or *legA3* (group XI) attenuated the growth defect of the Δ *sdhA* mutant (group IX) in *D. discoideum*. (B) The Δ *sdhA* Δ *lidA* mutant was more defective for growth than the Δ *sdhA* Δ *legA3* mutant in *A. castellanii*. (C) A Δ *mavP* Δ *legA3* mutant

grew as well as a Δ *legA3* single mutant in bone marrow-derived murine macrophages. (D) The Δ *mavP* Δ *legA3* mutant showed reduced growth relative to the Δ *legA3* mutant in *A. castellanii*. (A to D) Bacterial growth was monitored as described in Fig. 2. Data are representative of at least two independent experiments $\pm$ SD of three replicates.

specific set of Dot/Icm TSs. Similar to the partial defect in GFP-HDEL recruitment observed for the Δ *wipB* Δ *lidA* mutant (Fig. 2C), the residual growth of specific double mutants in *A. castellanii* or macrophages (Fig. 4, C and D) is consistent with existence of additional compensatory pathways promoted by other functional groups.

Selective pressures defining virulence protein repertoires. Maintaining an extensive repertoire of Dot/Icm TSs enables *L. pneumophila* to grow in a broad assortment of amoebal hosts (16). As it is common for many TSs to be present in only a subset of isolates (24), much of the selective pressure for preserving Dot/Icm TSs appears to be centered on maintaining sets of functions, rather than individual proteins. For example, activation of Rab1 appears to be a central feature of *L. pneumophila* pathogenesis. A key player in this event in the Philadelphia 1 strain is SidM, which acts at two levels to activate and recruit Rab1 to the LCV (25, 26), but is only encoded by a subset of clinical strains (24). Presumably, the presence of TSs such as AnkX, which activates Rab1 via a phosphocholine transfer mechanism (27), allows conservation of function. Therefore, activation of Rab1 is conserved without conserving specific proteins, or even the enzymatic activities they perform to activate this modulator of vesicle trafficking.

The accumulation of a large repertoire of Dot/Icm TSs to cope with host variation allows different combinations of proteins to promote growth, arming the bacterium with multiple mechanisms for accomplishing a single task as long as the appropriate host targets are present. This flexibility is limited, however, as it was possible to impair intracellular growth by disrupting more than one pathway (Fig. 1C). The host cell also restricts the degree of flexibility the bacterium can exert,

as the specific combinations of Dot/Icm TSs that were required for optimal growth varied between hosts (Fig. 4).

We have developed a strategy to elucidate the network of functional interactions central to *Legionella*'s manipulation of host cells. This approach defines sets of proteins that participate in common pathways and groups of proteins that modulate redundant virulence strategies, and predicts cell-biological defects on the basis of hierarchical clustering. For pathogens that employ redundant virulence mechanisms, similar to *L. pneumophila*, simple strategies such as the construction of strains with random sets of genetic lesions have failed to identify critical bacterial determinants of pathogenicity and have given no information on the interrelationships between members of a large group of proteins known to interact with the host cell. Our integrated approach can be applied to other bacterial pathogens, as well as other systems that have convergent biochemical pathways, and shows that it is now possible to define roles for individual proteins and their relative contributions to pathogenesis in different hosts. The utility of this strategy can be extended to any interface between two organisms involving a large number of interactions.

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Supplementary Materials

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A Universal Scaling for the Energetics of Relativistic Jets from Black Hole Systems

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Black holes generate collimated, relativistic jets, which have been observed in gamma-ray bursts (GRBs), microquasars, and at the center of some galaxies [active galactic nuclei (AGN)]. How jet physics scales from stellar black holes in GRBs to the supermassive ones in AGN is still unknown. Here, we show that jets produced by AGN and GRBs exhibit the same correlation between the kinetic power carried by accelerated particles and the gamma-ray luminosity, with AGN and GRBs lying at the low- and high-luminosity ends, respectively, of the correlation. This result implies that the efficiency of energy dissipation in jets produced in black hole systems is similar over 10 orders of magnitude in jet power, establishing a physical analogy between AGN and GRBs.

Relativistic jets are ubiquitous in the cosmos and have been observed in a diverse range of black hole systems spanning from stellar mass ($\sim 10 M_{\odot}$; $M_{\odot}$, solar mass) to supermassive scales ($\sim 10^5$ to $10^{10} M_{\odot}$), particularly in the bright flashes of gamma-rays [known as gamma-ray bursts (GRBs)] (1, 2), the miniature versions of quasars lurking in our Galaxy (known as microquasars) (3), and active galactic nuclei (AGN) (4, 5). Despite decades of observations at almost all wavelengths and consid-

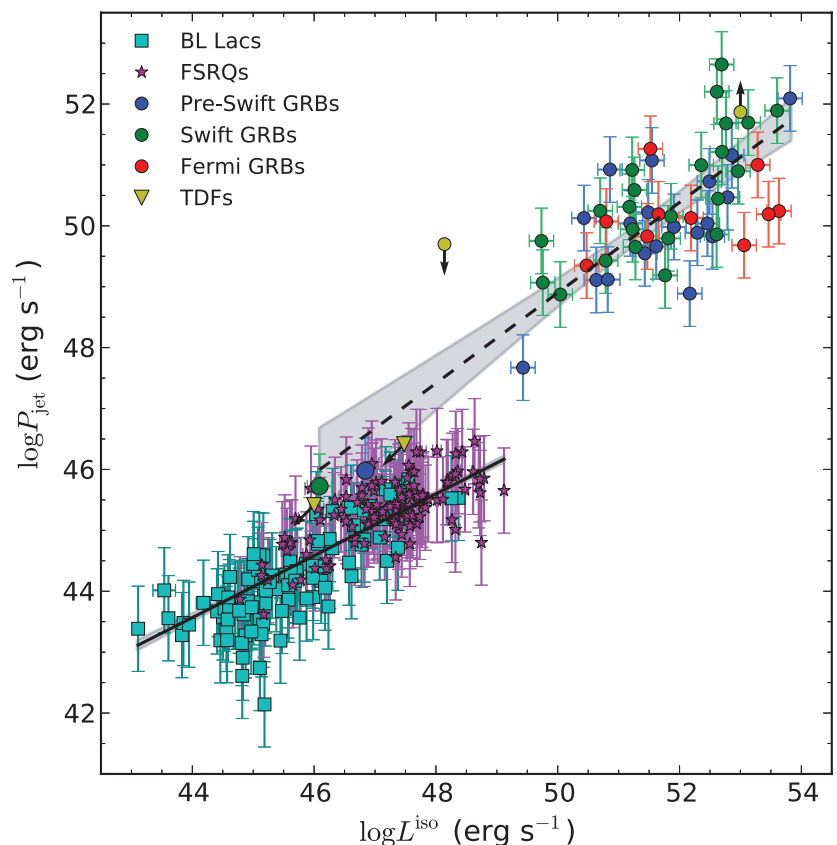
erable theoretical efforts, many aspects of black hole jets still remain mysterious, such as the mechanism(s) responsible for their formation and the nature of their energetics, as well as their high-energy radiation (6, 7). Jets and outflows from supermassive black holes have important feedback effects on scales ranging from their host galaxies to groups and clusters of galaxies (8). Hence, a better understanding of the physics of jets is required to have a more complete picture of the formation and evolution of large-scale struc-

tures in the universe and the coevolution of black holes and galaxies (9).

One outstanding question is how the jet physics scale with mass from stellar to supermassive black holes. Interestingly, there is evidence to suggest that jets behave in similar ways in microquasars and radio-loud AGN (10–12). However, a clear connection between AGN and GRBs has not yet been established, though recent work provides encouraging results (13, 14).

As a first step in understanding how the properties of jets vary across the mass scale, we focus on the energetics of jets produced in AGN and GRBs. Therefore, we searched the literature for published and archival observations that allow us to estimate the jet radiative output and the kinetic power for a sample of black hole systems in which the jet is closely aligned with our line of sight and characterized by a broad range of masses. For this reason, our sample consists of blazars—AGNs with their jets oriented

Fig. 1. Relation between the jet kinetic power and the isotropically equivalent gamma-ray luminosity for AGNs and GRBs. Error bars denote 1σ uncertainty. We fitted the two populations separately using a symmetric least-squares method (orthogonal bivariate correlated errors and intrinsic scatter with bootstrapping) (35). The blazar and GRB best-fit models correspond to the solid and dashed lines, respectively ($\log P_{\text{jet}} = A \log L^{\text{iso}} + B$). The best-fit parameters obtained for the blazars are $A = 0.51 \pm 0.02$ and $B = 21.2 \pm 1.1$; for the GRBs, $A = 0.74 \pm 0.08$ and $B = 11.8 \pm 4.1$. The scatter about the best-fit is 0.5 and 0.8 dex for the blazars and GRBs, respectively. The 2σ confidence band of the fits is shown as the gray shaded regions (barely visible for blazars). The two correlations do not agree at the $>5\sigma$ level. For illustrative purposes, we also include XRF 020903 and GRB 090423 (yellow circles), as well as the two recent tidal disruption flares (TDFs) detected with Swift, which are presumably due to the onset of relativistic jets from the tidal disruption of stars by supermassive black holes (36). We do not consider these sources in the statistics, because we only have limits on their luminosities. FSRQs, flat-spectrum radio quasars.



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toward Earth (15)—and GRBs, the spectral energy distributions of which are completely dominated by the jet due to beaming effects.

Fig. 2. Relation between the apparent gamma-ray luminosity and the beaming factor for blazars (left) and GRBs (right). We find correlation coefficient $r = -0.53$ and -0.56 for blazars and GRBs, respectively, indicating anticorrelations significant at the 3.6σ and 4.4σ levels, respectively. The solid lines correspond to the best-fit linear models obtained with the symmetric least-squares fit and are given by $f_b \approx 5 \times 10^{-4} (L_{49}^{\text{iso}})^{-0.39 \pm 0.15}$ and $\approx 0.03 (L_{49}^{\text{iso}})^{-0.24 \pm 0.06}$ for blazars and GRBs, respectively, where $L_{49} \equiv L/10^{49}$. The gray shaded region corresponds to the 1σ confidence band, and the blue and yellow regions are the 1σ prediction bands, which quantify the scatter about the best fits.

As a proxy of the jet bolometric luminosity, we used the observed gamma-ray luminosity L^{iso} , which is isotropically equivalent. To esti-

mate the kinetic power P_{jet} , we use extended radio luminosities for the blazars, whereas for the GRBs we relied on the afterglow measure-

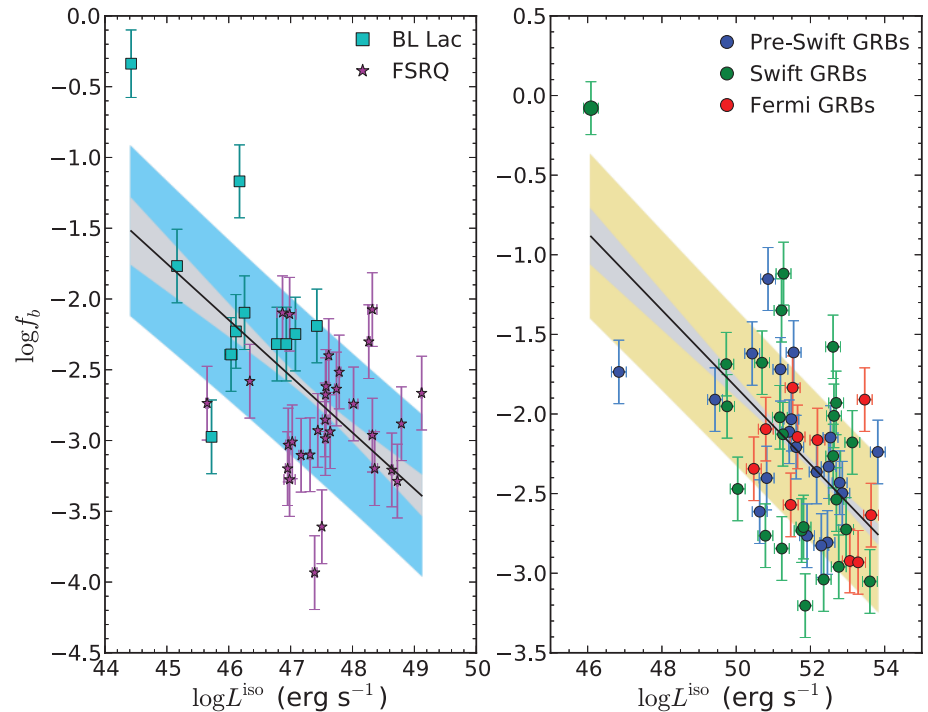
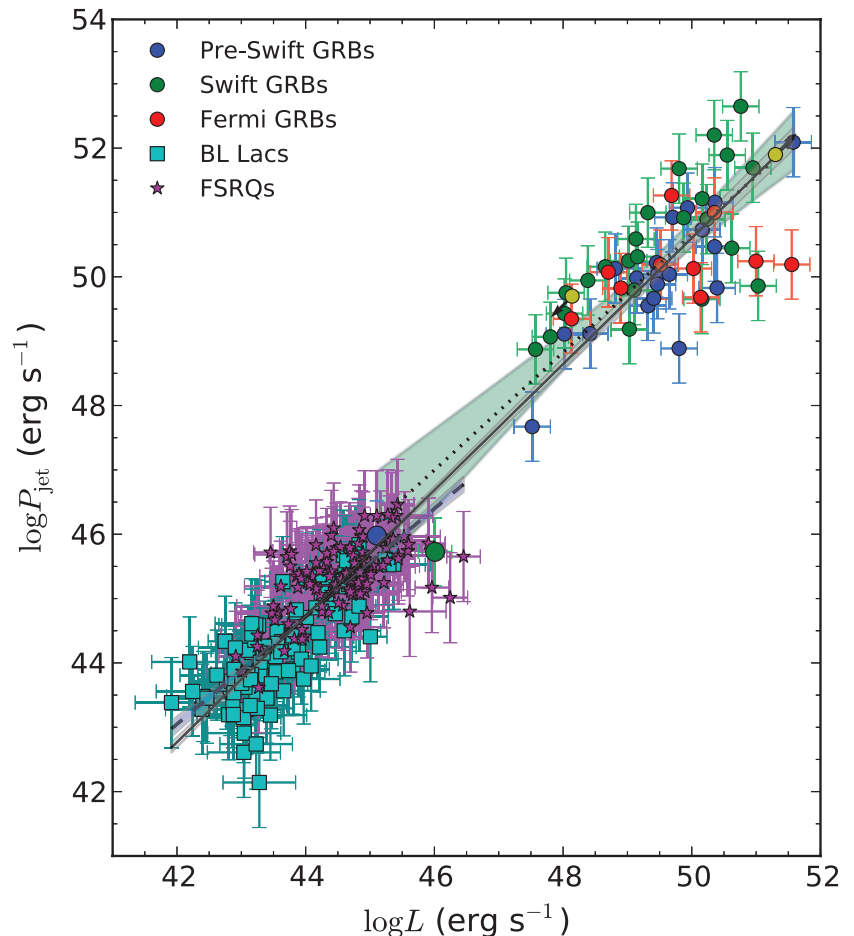


Fig. 3. Relation between the collimation-corrected gamma-ray luminosity $L = f_b L^{\text{iso}}$ and the kinetic power for AGNs and GRBs. The shaded regions display the 2σ confidence band of the fits. The blazar and GRB best-fit models (dashed and dotted lines, respectively) follow correlations that are consistent, within the uncertainties, with the best-fit model obtained from the joint data set (solid line). In other words, using L instead of L^{iso} leads to correlations for AGNs and GRBs that are consistent with each other (compare to Fig. 1). The best-fit parameters obtained from the combined data set are $\alpha = 0.98 \pm 0.02$ and $\beta = 1.6 \pm 0.9$, where $\log P_{\text{jet}} = \alpha \log L + \beta$. The scatter about the best-fit is 0.64 dex. The yellow data points correspond to XRF 020903 and GRB 090423, which we do not take into account in the statistics.



ments in radio or x-rays. Therefore, the availability of these observables restricted our sample to 234 blazars (106 BL Lacs and 128 flat-spectrum radio quasars) (see table S1) and 54 GRBs (49 long and 5 short GRBs, all with known redshifts z) (see table S2). For blazars, we estimated L^{iso} from the gamma-ray energy flux and the spectral index measured with the Fermi Large Area Telescope (LAT) (16). P_{jet} was estimated using an empirical correlation, which relates the Very Large Array (VLA) extended radio emission and the jet kinetic power (17, 18). For GRBs, $L^{\text{iso}} = E^{\text{iso}}(1 + z)/t_{90}$, where t_{90} is the burst duration and E^{iso} is the isotropically equivalent energy radiated during the prompt emission phase and measured with different telescopes (21 observed with either BeppoSAX, BATSE, HETE, HETE-2, or Integral; 24 with Swift Burst Alert Telescope; and 10 with Fermi). We computed P_{jet} as $P_{\text{jet}} = f_b E_k^{\text{iso}}(1 + z)/t_{90}$, where E_k^{iso} is the kinetic energy estimated from the radio (VLA) or x-ray (Chandra) luminosity during the afterglow phase using the standard afterglow model (19); $f_b \equiv 1 - \cos\theta$ is the “beaming factor”; and θ is the radiation cone half-opening angle, which is the same as the jet opening angle estimated from the jet break in the GRB afterglow light curve (20).

We first compared the relative trends of L^{iso} and P_{jet} for the blazar and GRB population separately (Fig. 1). The Pearson correlation coefficients of 0.85 and 0.8 obtained for blazars and GRBs, respectively, indicate a strong correlation within each group of sources. However, the $L^{\text{iso}}-P_{\text{jet}}$ trend is different for GRBs and blazars, as shown by the fits to the data (Fig. 1).

We computed the intrinsic luminosity L for GRBs and blazars by correcting L^{iso} for the opening angle or beaming factor f_b such that $L = f_b L^{\text{iso}}$. For GRBs, the beaming factor is computed from the jet opening angle θ_j as $1 - \cos\theta_j$ (21); for blazars, f_b is estimated as $1 - \cos 1/\Gamma$, where Γ is the bulk Lorentz factor of the flow, because AGNs obey $\theta_j < 1/\Gamma$ (22, 23). Although an estimate of θ_j is available for each GRB in the sample, Γ is only available for a subset of 41 blazars. Figure 2 shows an anticorrelation between L^{iso} and f_b for both GRBs and blazars with compatible indices when fit with a power law. Because θ is not available for the whole blazar sample, we used the power-law fit of L^{iso} versus f_b as an estimator for f_b .

As with L^{iso} and P_{jet} , L and P_{jet} are strongly correlated within the GRB and AGN samples (Fig. 3). However, they follow the same trend within the narrow uncertainties and the whole GRB and blazar sample can be fit adequately with a power law over 10 orders of magnitude in luminosity. Therefore, the relativistic jets in GRBs and blazars are consistent with obeying the relation $P_{\text{jet}} \approx 4.6 \times 10^{47} (L/10^{47})^{0.98} \text{ erg s}^{-1}$, within the measurement uncertainties. In other words, once “black hole engines” produce relativistic jets, they seem to do so maintaining the same coupling between the total power carried by the jet and power radiated away. This universal scaling for the energetics of jets is maintained across the mass scale, regardless of the different environments and accretion flow conditions around the compact object.

Figure 4 indicates that most of the jets in our sample dissipate at least 3% of the power car-

ried by the jet as radiation, and overall, they can radiate as much as 15%. This range of efficiencies is considerably higher than previous estimates for AGNs based on radio to x-ray luminosities (24, 25), but our results are in agreement with those obtained from blazar broadband spectral models (26, 27), as well as GRB afterglow studies (28–30). Efficient heating of electrons seems to be a universal property of relativistic magnetized shocks according to numerical simulations (31), which demonstrate that electrons retain $\geq 15\%$ of the preshock energy. If most of the postshock energy is radiated away, these theoretical results could pave the way to an understanding of the high dissipation efficiencies that we find.

Our results suggest that there is a single fundamental mechanism to produce relativistic jets in the universe. The analogy known to exist between microquasars and AGNs (3, 10, 11) can be extended to the gamma-ray bursts with the fundamental difference that, whereas AGNs and microquasars undergo recurrent activity, GRBs experience only one episode of hyperaccretion.

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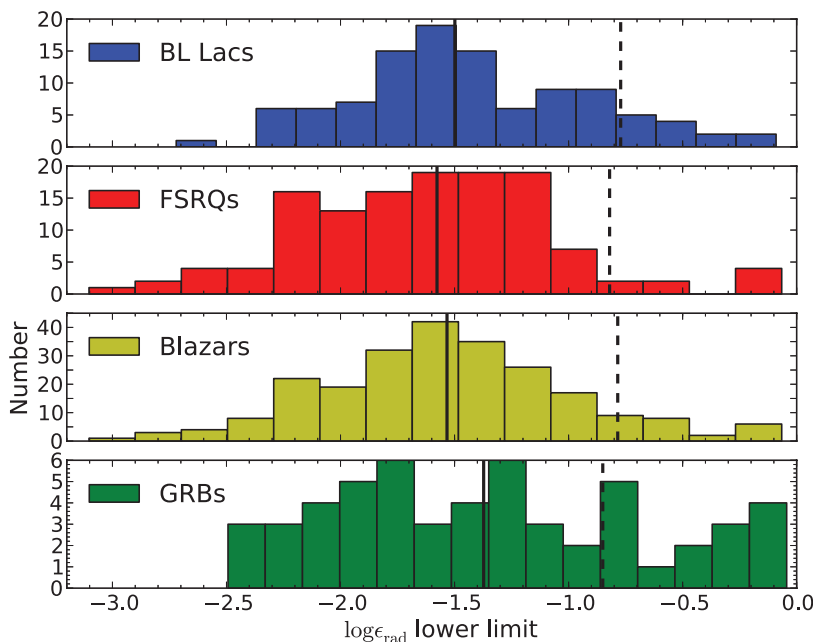


Fig. 4. Distribution of the 1σ lower limits on the jet radiative efficiency (the fraction of the total jet power that is converted to gamma rays) $\epsilon_{\text{rad}} \equiv L/(L + P_{\text{jet}})$ for AGNs and GRBs. The vertical solid lines indicate the median values of the lower limits; the dashed lines represent the median values of ϵ_{rad} for each sample. Most of the sources are characterized by $\epsilon_{\text{rad}} > 3\%$. The median efficiencies correspond to $\sim 15\%$, considering that these estimates are affected by ~ 0.5 to 0.7 dex uncertainties, on average.

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36. To include the tidal disruption flares in Fig. 1, we used the Fermi LAT upper limit (32) observed for Swift J164449.3+573451, and we assumed $L^{\text{iso}} \sim L_X$ for Swift J2058.4+0516 (33), where L_X is the observed x-ray luminosity. We assumed $P_{\text{jet}} \sim L_{\text{Edd}}$, where L_{Edd} is the Eddington luminosity based on the black hole masses estimated in (32–34). These luminosities and powers should be treated as upper limits.

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operated by the Jet Propulsion Laboratory, California Institute of Technology, under contract with NASA.

Supplementary Materials

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Supplementary Text

Fig. S1

Tables S1 to S4

References (37–75)

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Texture of Nanocrystalline Nickel: Probing the Lower Size Limit of Dislocation Activity

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The size of nanocrystals provides a limitation on dislocation activity and associated stress-induced deformation. Dislocation-mediated plastic deformation is expected to become inactive below a critical particle size, which has been proposed to be between 10 and 30 nanometers according to computer simulations and transmission electron microscopy analysis. However, deformation experiments at high pressure on polycrystalline nickel suggest that dislocation activity is still operative in 3-nanometer crystals. Substantial texturing is observed at pressures above 3.0 gigapascals for 500-nanometer nickel and at greater than 11.0 gigapascals for 20-nanometer nickel. Surprisingly, texturing is also seen in 3-nanometer nickel when compressed above 18.5 gigapascals. The observations of pressure-promoted texturing indicate that under high external pressures, dislocation activity can be extended down to a few-nanometers-length scale.

The plastic behavior of coarse-grained metals (with particle size >100 nm) is mainly controlled by the nucleation and motion of lattice dislocations. Plastic deformation by dislocation glide results in crystallite rotations, generating lattice-preferred orientation or texture. The anisotropic physical properties of a polycrystalline material are strongly related to the preferred alignment of its crystallites. In material science and engineering, texture control is essential in improving the strength and lifetime of structural materials (1). In Earth science, understanding texture development of minerals is important for interpreting seismic anisotropy in Earth's interior (2).

How plastic deformation occurs in nanocrystalline materials remains controversial (3–15).

Post-deformation analysis of compressed or indented nanocrystalline nickel does not indicate major dislocation debris (12), whereas dislocations are observed in 10-nm nickel and 9-nm platinum particles (13, 14). Deformation twinning and disclination have also been reported in several studies on nanocrystals (6, 9–11, 15). Although it is commonly believed that the intrinsic deformation behavior of nanomaterials arise from the interplay between defects and grain-boundary (GB) processes (11, 16), the precise trade-offs between these deformation mechanisms are still unclear, as is the effect of pressure on these different mechanisms. It has been proposed that below a critical length scale, the strength of nanometals would exhibit an inverse Hall-Petch size dependence because in the plastic deformation of fine nanocrystals, dislocation activity gives way to GB sliding, diffusion, and grain rotation (4, 5). In contrast, twin thickness has been found to affect the maximum strength of copper, implying that the plastic deformation of nanomaterials is not necessarily related to GB-mediated processes (9). Indeed, it has been proposed that dislocation nucleation governs material softening in nano-twinned metals (10).

Because of technical limitations, in situ observation of plastic deformation in ultrafine nanocrystals is difficult, precluding the direct exploration of mechanics at nanometer scales. Whether plas-

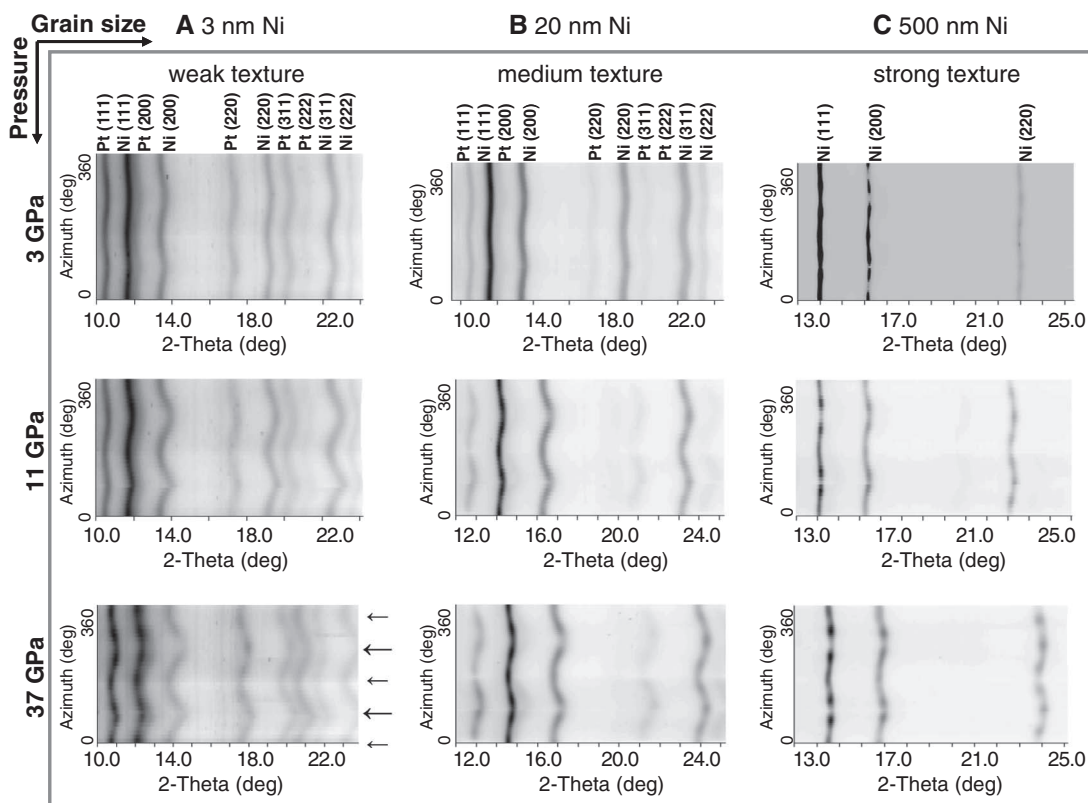
ticity in ultrafine nanocrystals is still generated by dislocations, how pressure affects the deformational regimes of nanoparticles, and how structural anisotropy is affected by size reductions are all unresolved questions. The effect of pressure on dislocations is complex in terms of both the high-pressure energetics of dislocation cores and their mobilities, with the net overarching effects of pressure being unclear (17). It has been observed that at high pressure, brittle materials such as oxides and silicates become ductile, even at room temperature (2). Our goal is to examine the interplay between pressure and particle size in determining when dislocation-mediated deformation processes predominate within nanoparticles and, correspondingly, what the lower size limit of dislocation activity is. In this work, radial diamond anvil cell (rDAC) x-ray diffraction (XRD) experiments (2) were used to make in situ observation of the texturing in stressed polycrystalline nickel of various mean particle sizes, from 500 nm down to 3 nm.

We deformed the nickel samples plastically in rDACs (fig. S1) (18). The XRD experiments were performed at beamline 12.2.2, Advanced Light Source (ALS), Lawrence Berkeley National Laboratory. The particle sizes of the nickel samples are 500 ± 45 nm, 20 ± 8 nm, and 3 ± 0.9 nm (fig. S2) (18), respectively. The relatively narrow size distributions allow the investigation of the size dependence of texturing. When shear stress is applied to polycrystals, individual crystals deform preferentially on slip planes. This results in crystal rotations that in turn lead to texture development (1). The radial diffraction images show variations in diffraction peak position with respect to the compression direction, indicating differential stresses in the material. They also display systematic intensity variations that can be used to deduce texture (Fig. 1 and figs. S3 to S6) (18). For instance, the diffraction intensity of the 500-nm nickel at 5.0 GPa is minimal in the compression direction for the (200) diffraction peak but maximal for the (220) peak (fig. S3). Diffraction intensity variations are seen in the 500-nm nickel above 3.0 GPa and in 20-nm nickel above 11.0 GPa, and more modest but resolvable intensity variations are observed in 3-nm nickel at pressures above 18.5 GPa. The variations in diffraction intensity can be best seen in the “unrolled diffraction” images recorded as a function of diffraction angle (Fig. 1).

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Fig. 1. Azimuthally (0 to 360°) unrolled diffraction images of (A) 3-nm, (B) 20-nm, and (C) 500-nm nickel. To enable direct comparison between runs, pressures are rounded (precise values are given in Fig. 2). The long thick arrows indicate the maximum compression direction, and the short thin arrows indicate the minimum compression direction. The curvature within the diffraction lines indicates that the sample is stressed. Texture is evident as the systematic intensity variations of the diffraction peaks along the azimuthal direction. Some images contain mixed diffraction from the nickel sample and the platinum pressure calibrant. The diffraction of 20-nm particles at 3 GPa and 3-nm particles at 37 GPa was taken with an x-ray wavelength of 0.4133 Å. For all other measurements, a wavelength of 0.4959 Å was used.



Rietveld refinement, implemented in the MAUD software (19), was used to analyze the differential stress, microstructure, and texture of the samples at each pressure. Texture is represented with inverse pole figures of the compression direction (Fig. 2 and fig. S7): These show the probability of finding the poles (normal) to lattice planes in the compression direction. The texture in the 500-nm nickel evolves at pressures as low as 3.0 GPa (Fig. 2 and fig. S8). Texture strength—the degree of lattice-preferred orientation (18)—increases to 2.4 multiples of random distribution (MRD) at 35.0 GPa. In the experiment with the 20-nm nickel, obvious texture starts to develop at 11.0 GPa with 1.7 MRD. Upon further compression to 38.5 GPa, the texture strength of the 20-nm nickel increases to 2.0 MRD. In the 3-nm nickel experiment, a weak but substantial texture (1.4 MRD) is observed upon compression above 18 GPa. The texture observations indicate that dislocation-mediated plastic deformation is still active in the 3-nm nickel when high external pressure is applied. It is known that dislocation glide on preferred slip systems gives rise to crystallographic texture, whereas grain rotation via GB sliding alone randomizes the grain orientation distribution (3). The GB activities are believed to be more active in smaller nanocrystals. The observed decrease of texture strength with decreasing particle size indicates that the texturing is not primarily derived from GB processes. Our separate measurements show that carbon-coated nickel nanocrystals, when deformed at high pressure, have similar texturing to pure

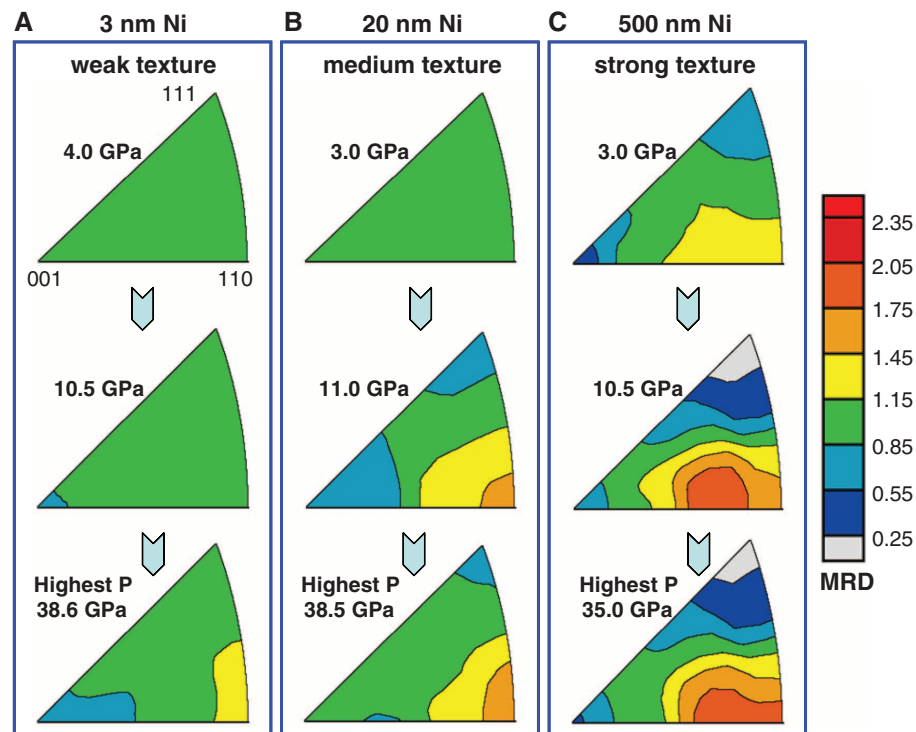


Fig. 2. Inverse pole figures of (A) 3-nm, (B) 20-nm, and (C) 500-nm nickel along the compression direction (normal direction). Equal area projection and a linear scale are used. Texture strength is expressed as MRD, where MRD = 1 denotes random distribution, and a higher MRD number represents stronger texture.

nickel particles of the same size (fig. S9), which strongly supports that the observed texturing is not arising from GB processes.

As shown in Fig. 1, at the same pressures the differential strain of the 3-nm nickel is higher than that of coarser particles. The larger curvatures

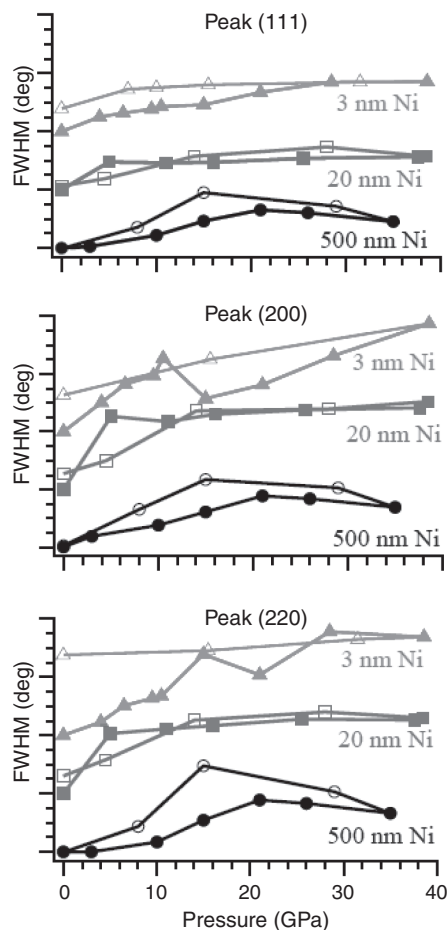


Fig. 3. The peak broadening of the nickel in compression relative to ambient pressure. The full width at half maximum (FWHM) is determined in terms of diffraction angle, 2θ . The curves are offset for clarity, and the small ticks represent 0.05° . For the FWHM determination, the signal is integrated over 10° around the compression direction. The solid symbols represent compression data, whereas the open symbols represent decompression data. The lines connecting the data points are guides for the eye.

of diffraction lines for smaller particles indicate higher elastic deformation and greater ability for the material to support the differential stress in the crystal plane without plastic deformation (20). From the lattice strain, shear stress can be estimated (18). According to the MAUD analysis, the differential stress on the 3-nm nickel is 8.0 GPa at an external pressure of 38.6 GPa, for the 20-nm nickel is 5.1 GPa at 38.5 GPa external pressure, and for 500-nm nickel is 2.9 GPa at 35.0 GPa external pressure. Differences in the level of dislocation activity are likely to account for these systematic variations. Lower dislocation contents and reduced mobility due to the enhanced dislocation-GB interaction in smaller particles could result in pinned dislocations, so elastic stress, built up in compression, is only minimally released by plastic deformation.

To obtain additional insights into the plastic deformation process, we studied the x-ray peak

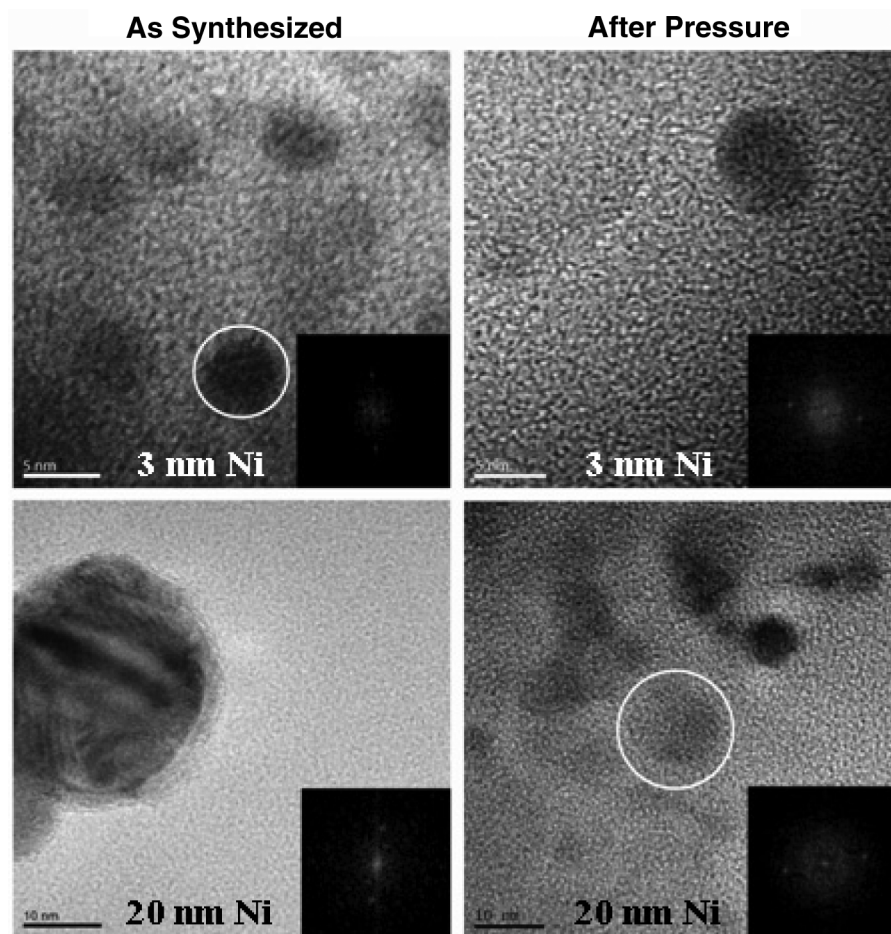


Fig. 4. Representative high-resolution TEM images of the (top) 3- and (bottom) 20-nm nickel particles as synthesized and as recovered from compression. The insets show the Fourier transforms of the particle images, indicating the absence of lattice defects before and after pressure. When more than one particle is present in the image, the inset corresponds to the circled particle. In the image of the 20-nm nickel particles after compression, only the circled particle was isolated; the other particles were not isolated and were damaged under the electron beam.

broadening of the samples at high pressures (Fig. 3). It is known that peak broadening is generated by several factors, including instrumental broadening, grain size effects, and stress (microstrain)-induced broadening (21). A standard material, LaB_6 , was used to characterize the instrumental broadening. The peak broadening of unstressed samples was used to confirm the particle size that was measured with transmission electron microscopy (TEM) and scanning EM (Fig. 4 and fig. S2). It has been verified that there is usually no particle coarsening and associated peak width reduction under compression if there are no crystallite domain changes, as are produced by phase transformations (22). Thus, it is reasonable to ascribe the observed peak broadening to local stresses produced by inhomogeneous strain (9).

The evolution of peak broadening of the three stressed samples shows substantial differences (Fig. 3). The broadenings of the three peaks of the coarse particles have similar pressure dependencies, whereas the broadenings of the three peaks of 3-nm nickel evolve differently with pressure. This difference likely arises from varying

sensitivities of the differently sized particles to particle shape change. Pressure-induced particle shape changes in fine nanocrystals could result in peak width changes (23). Strain profiles in compression also affect peak broadening. In the 500-nm nickel, the peak broadening increases to a maximum as pressure is increased to ~ 21.0 GPa, indicating that peak broadening in this pressure range mainly comes from the elastic strain. Upon further compression, the peak broadening decreases slightly. Although the observation of texture in 500-nm nickel indicates the existence of dislocations above 3.0 GPa, the increased plastic strain does not dominate the local strain up to a pressure of 21.0 GPa. In the 20-nm nickel, there is not much enhancement of peak broadening in compression above ~ 5 GPa, indicating that plastic strain dominates the peak broadening, which suggests that other mechanisms probably dominate the plastic deformation before the onset of texturing. The peak broadening in the 3-nm particles is substantially less reversible on decompression than those of the two coarser samples (Fig. 3). The straightness

Table 1. Calculated critical shear stress needed for nucleation of dislocations (Eqs. 1 and 2) and the measured shear stress at the onset of texturing in nickel. For the calculations, $b_f = 0.487$ nm, $b_p = 0.244$ nm (7), $G = 76$ GPa (24, 26), $\gamma = 120$ to 130 mJ/m² (27), and $\nu = 0.31$ (26).

Type of dislocations	Particle size of nickel					
	3 nm		20 nm		500 nm	
	Edge (GPa)	Screw (GPa)	Edge (GPa)	Screw (GPa)	Edge (GPa)	Screw (GPa)
Critical shear stress for nucleation of a full dislocation (theoretical)	12.3 (2.8*)	37.0	1.8	5.4	0.1	0.2
Critical shear stress for nucleation of a partial dislocation (theoretical)	6.7	19.1	1.4	3.3	0.5	0.6
Shear stress at the onset of texturing (this experiment)	2.4		1.7		0.2	

*When dislocation-GB interactions are considered.

of the unrolled diffraction lines of the quenched sample indicates that the residual stress is negligible. GB-mediated plastic deformation does not change the crystalline domain size in the interior of the particles. Thus, the irreversibility of peak broadening in the 3-nm nickel likely originates from pressure-induced particle shape change: A modest change in shape is consistent with our TEM images (fig. S10). For comparison, the crystallographic dimension change of coarser particles due to particle shape changes may be small relative to their particle size and thus may not much affect their peak width.

Nonhydrostatic compression provides the shear stress that generates dislocation nucleation. Although the textures formed at high pressure are quenchable (figs. S3 to S5), dislocations usually cannot remain in nanocrystals when no external shear stress sustains them, which is confirmed with TEM observations (Fig. 4). The TEM measurements indicate that the nickel nanoparticles remain largely defectless single crystals after high-pressure compression. Most nickel nanoparticles after compression do appear less spherical than do precompression particles (fig. S10), and this likely arises from the nonhydrostatic pressure-induced particle shape change.

The physics governing the observed pressure-promoted texturing in nanocrystals can be understood by considering the effect of pressure on dislocations. According to dislocation theory (6, 24), with the approximation that the source size is equal to the particle size (D), the critical shear stress needed to nucleate a full dislocation is

$$\tau_f = \frac{2aGb_f}{D} \quad (1)$$

and that needed to nucleate a partial dislocation is

$$\tau_p = \frac{2aGb_p}{D} + \frac{\gamma}{b_p} \quad (2)$$

where b_f and b_p are the Burgers vectors of the full and partial dislocations, respectively; G is the shear modulus; and γ is the stacking fault energy. The factor a is taken to be 0.5 for edge dislocations and 1.5 for screw dislocations. As shown in Table 1, the critical shear stress for dislocation nucleation increases dramatically with

decreasing particle size. The measured shear stress of 500- and 20-nm nickel at the onset of texturing is quite close to the theoretically predicted values from Eqs. 1 and 2, whereas the values are discrepant for the 3-nm particles. This discrepancy is probably because the interaction of dislocations with grain boundaries is not considered in this theoretical model (6). Shan *et al.* used this model to estimate that the critical size (d_c) for nickel ranges from ~11 to 22 nm (7). The observed texturing in the 3-nm nickel suggests that under pressure, the critical size for the deformation mechanism crossover shifts to substantially smaller sizes. In small nanocrystals, dislocations are emitted and absorbed mainly at grain boundaries, and dislocation-GB interaction is highly significant for the smallest grain sizes. A simple estimation (25) of the interaction of an edge dislocation with grain boundaries can be produced from $\tau_f = \frac{Gb_f}{4\pi(1-\nu)l_{ds}}$, where ν is the Poisson's ratio and l_{ds} is the equilibrium separation between a dislocation and a grain boundary. This yields a shear stress of ~2.8 GPa for keeping an edge dislocation at the center of 3-nm nickel particles, which is in very good agreement with our measured shear stress at the onset of texturing in the 3-nm particles (Table 1). Thus, the strength of the smallest nanoparticles appears to be critically dependent on dislocation-GB interactions.

These results emphasize the importance of rDAC XRD experimentation in assessing plastic deformation in nanomaterials. Through a combination of textural and line-broadening analysis, the changing contribution of dislocation-associated deformation mechanisms can be assessed as a function of both pressure and sample particle size. Our results demonstrate that dislocation-mediated deformation persists to smaller particle sizes than anticipated. Such in situ high-pressure textural studies thus provide the means to investigate deformation mechanisms and help constrain the fundamental physics of deformation at the nanoscale.

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Small Gold Clusters Formed in Solution Give Reaction Turnover Numbers of 10^7 at Room Temperature

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Very small gold clusters (3 to 10 atoms) formed from conventional gold salts and complexes can catalyze various organic reactions at room temperature, even when present at concentrations of parts per billion. Absorption and emission ultraviolet-visible spectroscopy and matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry revealed that, for example, the ester-assisted hydration of alkynes began only when clusters of three to five gold atoms were formed. The turnover numbers and turnover frequencies associated with these catalyzed reactions can be as high as 10^7 and 10^5 per hour, respectively.

The current interest in gold catalysis stems from the discovery 25 years ago that nanosized gold can catalyze the hydrochlorination of acetylene and the oxidation of carbon monoxide at room temperature (1, 2). Gold nanoparticles (AuNPs) can activate O_2 , H_2 , sp^3 , sp^2 , and sp . C–X bonds (X = C, H, halogen, boronic acid, etc.), among others, giving access to new reaction pathways (3–5). Gold salts and complexes have catalytic activity in solution, particularly for unsaturated C–C bonds; new routes for organic synthesis have taken advantage of this activity (6, 7). In particular, readily available Au(I) and Au(III) chloride salts or complexes have been used as Lewis catalysts for many of these homogeneous reactions. However, their ubiquity has puzzled chemists because these catalysts perform similarly irrespective of the gold oxidation state. Some authors have speculated that Au(III) is reduced to Au(I) under reaction conditions and that the latter is the true catalyst; others have attributed this similar catalytic performance to the disproportionation of Au(I) to Au(III) and Au(0) (8–10). Here, we show that different Au(I) and Au(III) salts or gold complexes form 3- to 10-atom gold clusters in solution at room temperature and can act as extremely active catalysts, with turnover number (TON) values up to $\sim 10^7$ and turnover frequency (TOF) values up to $\sim 10^5$ hour $^{-1}$. We present results for two types of representative Au-catalyzed reactions in solution: the ester-assisted intermolecular hydration of alkynes and the bromination of arenes (11–13). Efficient formation of these very small gold clusters in diluted suspensions achieved a catalytic activity nearly five orders of magnitude higher than those previously reported.

Several di- and triatomic gold(I) cationic complexes have been successfully isolated (14) and used as catalysts for particular reactions (15–18), as have multiatomic gold centers and well-defined AuNPs (19–21). However, the sub-nanosized regime remains little explored in catalysis, despite results suggesting that multiatomic gold entities with sizes intermediate between well-defined gold complexes and AuNPs are potential active species (22, 23).

When catalytic species form during a reaction, a reaction-induction period can generally be observed, provided that the rate of formation of the catalyst is slower than the rate of the reaction. No reaction-induction periods are generally found for Au-catalyzed homogeneous reactions, even though there are several examples in which the active species is not the

original source of gold (4, 7). The absence of an induction period can be explained by assuming that the active species is formed very quickly under the nominal amounts of Au used (1 to 5 mol%) and cannot be detected. However, if the amount of precursor is reduced to a level where the formation of the active gold species is measurable, then the catalytically active species and the rate of the reaction can be decoupled. To check this hypothesis, we carried out the ester-assisted hydration of alkynes in the presence of different amounts of Au(I) and Au(III) salts and, after optimization, performed kinetic experiments at 100-ppm concentrations of gold. The results in Fig. 1B show an induction period followed by a similar reaction rate for AuCl and HAuCl $_4$, indicating that the real gold active species must be formed before the catalysis starts and could be the same regardless of the nature of the gold salts used. Note that low yields of product 4 were observed when no gold was introduced in the reaction and that a similar kinetic profile was found when Au(OH) $_3$ was used as the gold source at 100 ppm; hence, this induction period was not due to autocatalytic or exothermic processes.

Because the ester-assisted hydration of alkynes has been reported with gold(III) salts and gold phosphine complexes at 1 to 5 mol% (10, 11), we also tested complexes AuPPh $_3$ NTf $_2$ 5 and AuPPh $_3$ Cl 6 as catalysts (Ph, phenyl; Tf, triflyl) but at low concentration (0.1 mol%). The evolution of the complexes was monitored by 1H and ^{31}P nuclear magnetic resonance (NMR) spectroscopy (fig. S1) (24). Decomposition of these complexes should give the diphosphine adduct Au(PPh $_3$) $_2$ X 7 and AuX, and indeed, the quantitative formation of complex 7 was observed after adding alkyne 2.

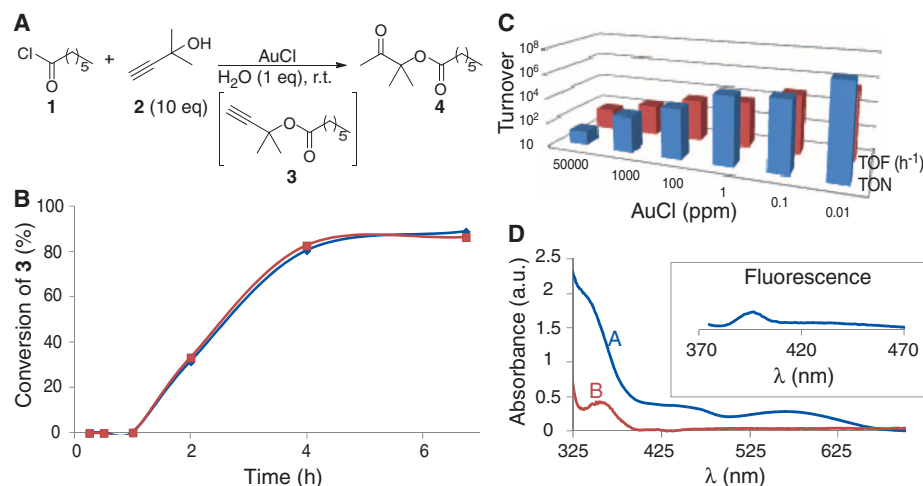


Fig. 1. Ester-assisted hydration of in situ–formed alkyne 3. (A) Structures of 1, 2, 3, and 4. (B) Plot-time conversion for AuCl (squares) and HAuCl $_4$ (diamonds) at 100 ppm, after correction with the blank experiment. (C) Turnover number (TON) and turnover frequency (TOF) for different amounts of AuCl, calculated as moles of 4 formed per mole of AuCl at final conversion (TON) and as the initial reaction rate after the induction time per mole of AuCl (TOF). The final yield of 4 is >90% in all cases, except for 0.01 ppm, where it is ~50%. (D) Absorption measurements (a.u., arbitrary units) for the hydration of 3 containing the Au active species along the induction time (A) and when the reaction proceeds (B) with the corresponding fluorescence (inset, irradiated at 349 nm).

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Because the formation of ketone **4** occurred while **7** was present in the reaction mixture, it might be initially concluded that **7** was the active gold catalyst. However, when **7** was independently prepared and used as catalyst, the reaction did not take place. Thus, if **7** is not the catalytic species, the AuX salt is the only species involved in the catalysis, which connects with the results described above for gold(I) chloride salts (25).

The reaction was carried out with different concentrations of AuCl; the results are given in Fig. 1C. Gold formed an active catalyst at concentrations of 10 ppb, yielding a TON value of $\sim 10^7$. To our knowledge, this value is substantially greater than any value reported for a non-enzymatic catalyst at room temperature.

We investigated the possible generation of AuNPs during the induction period, and their evolution during the reaction, with the use of matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry and ultraviolet-visible (UV-vis) spectroscopy. We took samples periodically while checking the progress of the reaction by gas chromatography (GC). Mass fragments and fragmentation patterns indicated that very small Au clusters formed in a few seconds and that these clusters did not aggregate to more than 13 atoms (26) at any moment [<2500 daltons; for MALDI-TOF spectra, mass analyses, and formula assignment, see fig. S2 (24)]. Initially, clusters were formed mainly by 5 to 9 Au atoms, which rearranged over time. The hydration of **3** started only when species with

3 to 5 gold atoms ($\text{Au}_3\text{-Au}_5$ clusters) predominated. Absorption UV-vis spectroscopy (Fig. 1D) confirmed these observations: The original absorption band for AuCl rapidly decomposed into three new bands at ~ 350 , 450, and 570 nm, which correspond to the jellium model predictions for 3 to 5, 8 to 10, and 13 gold atoms, respectively, and finally collapsed into a main band at ~ 349 nm (4 to 5 Au atoms) when the reaction started and proceeded (27). Fluorescence measurements confirmed the energetic absorption of the Au cluster at the band gap level (see below) (23, 27). After 6 hours ($\sim 80\%$ conversion), the reaction practically stopped, and in this situation, few $\text{Au}_3\text{-Au}_5$ clusters were observed. These results would indicate that for the ester-assisted hydration of alkynes, $\text{Au}_3\text{-Au}_5$ clusters can catalyze the reaction with very high turnover frequencies.

The results in fig. S3 show that the presence of H_2O was not necessary to form the $\text{Au}_3\text{-Au}_5$ clusters because the reaction started just after their addition. In contrast, when solvent **2** was absent, the Au nanoclusters were not observed even after 2 hours. However, if solvent **2** was added after 2 hours, the reaction started after an induction period. In other words, the $\text{Au}_3\text{-Au}_5$ clusters were only formed in the presence of alkyne **2**, and when they were formed, the induction period disappeared; hence, these clusters were very probably the catalytic active species. It is not surprising that very small gold clusters possess Lewis acidic character; recent studies have shown that the so-called molecular clusters

(fewer than 20 atoms) present an available low unoccupied molecular orbital (LUMO) for nucleophilic interactions (28, 29). The role of HCl was also investigated with reactions performed with different acids and chloride salts. The reaction also proceeded in HBr, but no reaction occurred when alkyl ammonium or sodium chloride salts were used instead, which indicates that a low pH was key for the catalysis and that chlorides were apparently not necessary for the reaction to proceed.

The generality of the reaction was studied; the results are presented in Fig. 2. Alkyl and aryl propargyl esters with different functionalities were transformed to the corresponding ketones in good yields and high selectivity with AuCl concentrations of 200 ppm to 10 ppb.

These results indicate that very small Au clusters of 3 to 5 atoms were formed from different Au sources at room temperature in acidified propargyl alcohol, and that these clusters could catalyze the ester-assisted intermolecular hydration of alkynes with catalytic efficiencies up to five orders of magnitude greater than those previously reported. In addition, the work described here rationalizes the similar catalytic efficiency observed for Au(I) and Au(III) compounds. To determine whether small gold clusters, rather than cationic gold, were the active species for other homogeneous reactions, we tested the AuCl_3 -catalyzed bromination of arenes (11). This reaction has been reported with catalytic amounts of AuCl₃ ranging from 1 to 0.01 mol%, and the authors ascribed this high catalytic efficiency to the Lewis acidity of Au(III). However, if it is not Au(III) but the small clusters formed that are responsible for the catalytic activity, the less acidic AuCl should also work efficiently. The results in Fig. 3B show that when using AuCl at ppm concentrations under the reported reaction conditions—namely, dichloroethane (DCE) as a solvent at room temperature (11)—a catalytic efficiency similar to that with AuCl₃ after an induction period was observed, and UV-vis measurements (fig. S4) indicate that small clusters of 7 to 9 atoms were responsible for the catalysis. Thus, it seems that different-sized clusters catalyze the two homogeneous reactions studied here.

The formation of these $\text{Au}_7\text{-Au}_9$ clusters was slower in DCE than in alkyne **2**, so the bromination of arenes in alkyne **2** should proceed independently of the ester-assisted hydration of alkynes, given that the catalytic gold clusters formed at different rates. To test this hypothesis, we performed the one-pot hydration of **3** in the presence of the reactants for the bromination **16** and **17**, and the results in Fig. 3C show that the bromination of **16** rapidly started according to the early formation of $\text{Au}_8\text{-Au}_9$ clusters in acidified solvent **2** (fig. S2). However, as the concentration of $\text{Au}_3\text{-Au}_5$ clusters increased, the number of $\text{Au}_5\text{-Au}_9$ clusters decreased and the formation of **16** was inhibited, so the hydration of **3** was the only reaction taking place. This effect was also observed if the reactants for the

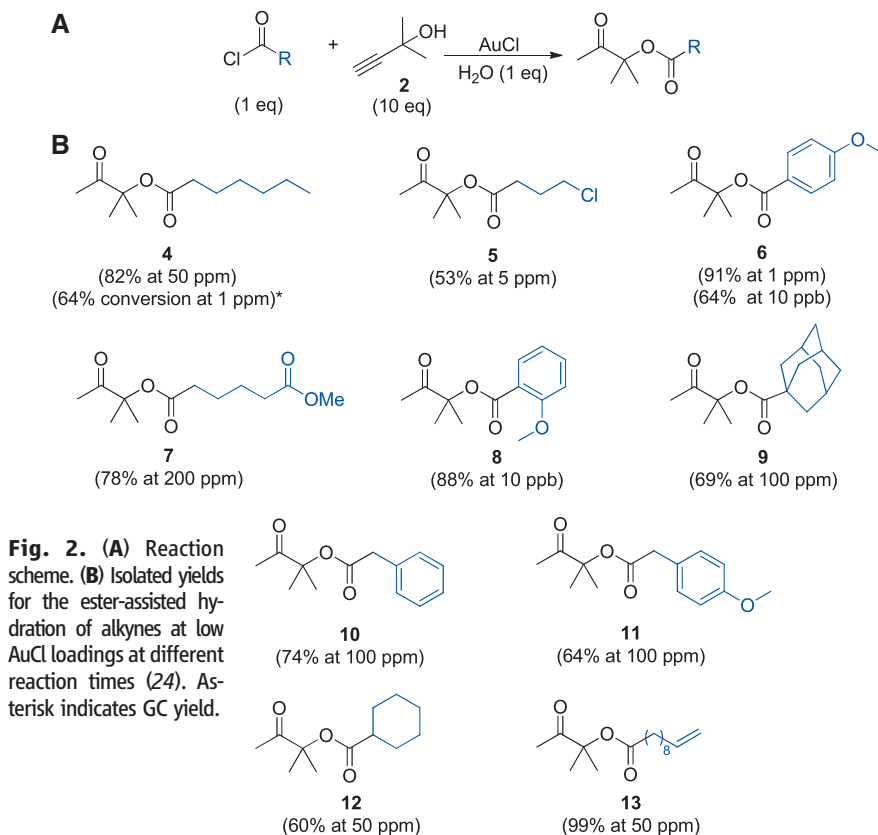


Fig. 2. (A) Reaction scheme. **(B)** Isolated yields for the ester-assisted hydration of alkynes at low AuCl loadings at different reaction times (24). Asterisk indicates GC yield.

hydration, **1** and water, were added after 1.5 hours of reaction time (Fig. 3D). Accordingly, no bromination occurred if the reactants **16** and **17**

were added after 1.5 hours reaction time when no Au₈-Au₉ clusters were present (Fig. 3E). These results demonstrate that different small Au clus-

ters catalyze different reactions and can be differentiated by kinetic experiments.

To further confirm these findings, we independently synthesized Au₅ and Au₈ clusters stabilized on the dendrimer Poly(amineamide-ethanol) (PAMAM-OH) (**30**). The results in Fig. 4 show that Au₅-PAMAM at ppm concentrations did indeed catalyze the ester-assisted hydration of alkynes, whereas Au₈-PAMAM preferentially catalyzed the bromination reaction, with no induction time in both cases. However, the catalytic efficiency of the gold clusters when supported on the dendrimer was at least two orders of magnitude lower than formed *in situ* in the propargyl alcohol **2**.

A systematic study of the cluster formation in different solvents and acids revealed that very small gold clusters formed under a variety of reaction conditions, with a size distribution controlled by the nature of both the solvent and the acid, as determined by UV-vis spectroscopy (fig. S5). These results imply that very small gold clusters can be formed under different experimental conditions and can be used in other catalytic processes.

Regardless of the gold salt [AuCl, AuCl₃, Au(OH)₃] or gold complex (AuPPh₃Cl, AuPPh₃NTf₂) used, we observed rapid decomposition during the reaction to give gold clusters preferentially formed by 5 to 13 atoms. These clusters formed new Au₃-Au₅ clusters. Only when a critical concentration of the last clusters formed did the ester-assisted hydration of alkynes occur. However, when Au₃-Au₅ clusters were added at time zero, the ester-assisted hydration of alkynes immediately started with no induction period. With a second reaction (i.e., bromination of *p*-dimethoxybenzene), no induction period was observed with gold salts in acidified propargyl alcohol **2**, indicating that the larger Au₅-Au₉ clusters initially formed are active for this reaction. The bromination of *p*-dimethoxybenzene readily stopped when the Au₅-Au₉ clusters were transformed into Au₃-Au₅ clusters. When both reactions were performed simultaneously, the bromination of *p*-dimethoxybenzene readily started, but no hydration reaction was observed. After ~2 hours,

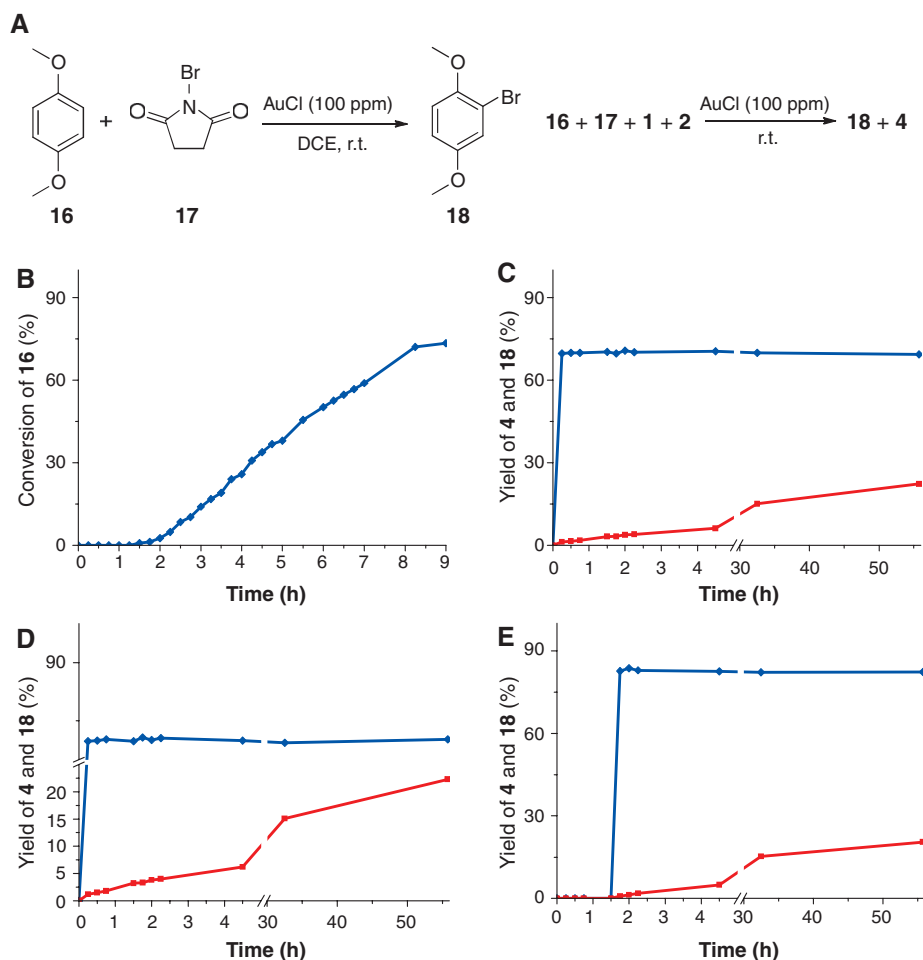
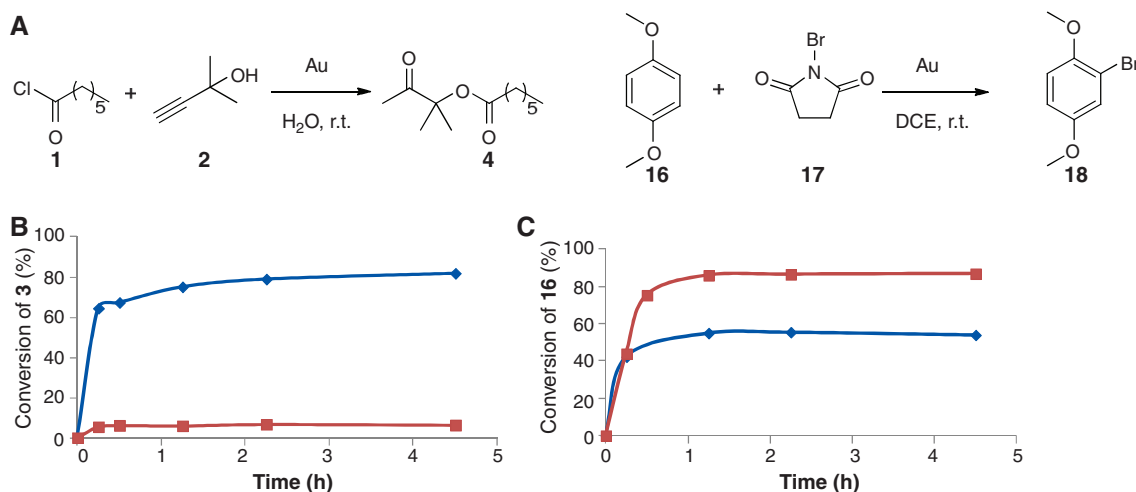


Fig. 3. (A) Studied reaction schemes. (B to E) Plot-time conversion for the bromination of arene **16** (B) and for the same reaction in the presence of the reactants for the ester-assisted hydration of alkyne under the following reaction conditions (bromination, diamonds; hydration, squares): (C) All the reactants are added from the beginning. (D) The reactants **2**, **16**, and **17** are added from the beginning; at 1.5 hours of reaction time, **1** and 1 equivalent of water are added to start the hydration reaction. (E) The reactants **1**, **2**, and water are added from the beginning; at 1.5 hours of reaction time, **16** and **17** are added.

Fig. 4. (A) Studied reaction schemes. (B and C) Plot-time conversion for the ester-assisted hydration (B) and for the bromination of *p*-dimethoxybenzene (C) with Au₅-PAMAM (diamonds) and Au₈-PAMAM (squares).



the bromination reaction stopped and the hydration rapidly proceeded, which reflected the transformation to a different type of clusters in solution. An independent synthesis of the different clusters and their use as catalysts confirmed the nature of the catalytic species. Our results may lead to more efficient Au catalytic systems and to a greater understanding of the chemistry of subnanosized particles in general.

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Supplementary Materials

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Enantioselective Heck Arylations of Acyclic Alkenyl Alcohols Using a Redox-Relay Strategy

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Progress in the development of asymmetric Heck couplings of arenes and acyclic olefins has been limited by a tenuous understanding of the factors that dictate selectivity in migratory insertion and β -hydride elimination. On the basis of key mechanistic insight recently garnered in the exploration of selective Heck reactions, we report here an enantioselective variant that delivers β -, γ -, or δ -aryl carbonyl products from acyclic alkenol substrates. The catalyst system imparts notable regioselectivity during migratory insertion and promotes the migration of the alkene's unsaturation toward the alcohol to ultimately form the ketone product. The reaction uses aryldiazonium salts as the arene source, yields enantiomeric products from opposite starting alkene configurations, and uses a readily accessible ligand. The racemic nature of the alkenol substrate does not bias the enantioselection.

Alkenes that engage in migratory insertion typically require an adjacent functional group to polarize the double bond for site-selective addition and enhanced reactivity (1–3). A classical and long-studied example is the Heck reaction, wherein an organometallic species derived from an aromatic group (Ar) is added to an alkene **A** followed by β -hydride elimination in **B** to formally replace a carbon–hydrogen bond with a carbon–carbon bond (Fig. 1A) (4–7). The alkene used in these reactions is generally electron-deficient through conjugation. An

electronically biased alkene of this type is necessary for two reasons: First, the charge distribution in **A** dictates site selectivity of the alkene insertion into the metal–Ar bond, and, second, these simple substrates oblige β -hydride elimination from **B** of a single C–H bond in the formation of **C** (8). Unfortunately, the use of biased substrates fundamentally limits the breadth of transformations available (9–11), motivating discovery of methods wherein the outcomes for both the migratory insertion of unactivated alkenes into aryl organometallic reagents, as well as elimination from complexes with different β -C–H bonds are predictable (4, 12, 13). In this context, we report a Pd-catalyzed enantioselective redox-relay Heck reaction of acyclic, alkenyl alcohols where the addition of the organometallic to the alkene is

coupled with oxidation of the alcohol by migration of the metal through the alkyl chain (Fig. 1, B and C) (14, 15). This method establishes chiral centers remote from the resultant carbonyl as highlighted by the ability to synthesize β -, γ -, or δ -aryl carbonyl chiral building blocks in high enantioselectivity from the corresponding racemic acyclic alkenol substrates.

The inspiration for this approach arose from the observation that an allylic alcohol substrate **J**, when submitted to a Heck reaction under conditions developed within our laboratory (13), gives rise to about equal amounts of ketone **L** and styrenyl product **M** (Fig. 1D). This is an outcome consistent with previous reports describing the use of allylic alcohols, although in some cases improved selectivity for products of type **L** are observed (10, 14, 15). This product distribution implies that the carbinol (H_C) and benzylic (H_B) C–H bonds in **K** are electronically similar and thus competitive in the key C–H bond cleavage event. We hypothesized that an enantioselective variant might be achieved by submitting 1,2-disubstituted allylic alcohol substrates to a system incorporating a chiral catalyst (Fig. 1B). This strategy would require the discovery of a catalyst sensitive to the subtle electronic differences between the carbons α and β to the alcohol in **E** and capable of distinguishing between electronically similar C–H bonds on the basis of their relative steric environments. We thought that the judicious choice of a bulky ligand would promote the required selective β -hydride elimination event and that modulating the electronic nature of the metal center would enable discrimination between subtly different alkene carbons in the insertion step.

The process leading to the ketone product requires migration of the alkene's unsaturation

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toward the tethered alcohol, leading to its oxidation (16, 17). This relay of redox information is attractive for uses in synthesis because it simultaneously promotes functionalization at multiple carbons within the molecule and, more importantly, allows us to envision expansion of the scope to substrates wherein the alcohol and the alkene are separated by additional carbon atoms. Such scope expansion would provide a method for setting a chiral center remotely from the newly formed ketone (Fig. 1C). Mechanistically, the process would require the transposition of the metal, after initial migratory insertion yielded **G**, whereby β -hydride elimination to form **H** followed by reinsertion would produce **I**. Repetition of these steps would ultimately form the ketone product effectively by walking Pd stepwise through the β - and α -carbons relative to the alcohol along the alkyl chain. To favor migration over dissociation of the metal from the alkene, we hypothesized that an electrophilic catalyst produced under mild conditions would be required to promote the binding and insertion of **H** (4, 18). This suggested the use of aryldiazonium salts as the arene source (13, 19, 20). However, these reagents have not been reported in enantioselective carbon–carbon bond-forming reactions of this sort because of their likely incompatibility with common, chiral phosphine-based ligands (20, 21).

Despite this lack of precedent, we investigated the use of chiral pyridine oxazoline (PyrOx) ligands (22–25), which were hypothesized to be compatible with aryldiazonium salts because of their reduced Lewis basicity relative to phosphine ligands (Fig. 2). These ligands were attractive for two major reasons: First, they are easily synthesized (normally two steps from commercially available materials) in a modular manner, allowing us to take advantage of our recently reported multidimensional parameter-based optimization protocol (26, 27), and, second, the ligands have two coordination sites with distinct electronic properties, which should allow for a well-defined coordination environment envisioned to be required for effective asymmetric catalysis (24, 28, 29). Therefore, a nine-membered ligand library was prepared and evaluated for efficacy in the redox-relay enantioselective Heck reaction of the simple racemic allylic alcohol **2a** and aryldiazonium salts. These experiments rapidly revealed that ligand **1a** was optimal in terms of rate and enantioselectivity [reported as enantiomeric ratio (er) in table S1]. However, a 5- CF_3 variant called **1b**, predicted by the mathematical model to function similarly, was selected for economic reasons. Further modest optimization of the reaction conditions resulted in a transformation that delivers arylated ketone products in generally high enantiomeric ratios (Fig. 3).

Submission of the simple substrate, **2a**, to coupling with electron-poor diazonium salts on a 0.5 mmol scale gave high yields (**3a** and **3b**) with good enantioselectivity (Fig. 3). Improved er (**3c**, 97:3) was observed when a substrate bearing a bulkier group on the saturated side of the

allylic alcohol was submitted. This trend continued because generally higher enantioselectivity was observed with greater substitution on the saturated side of the allylic alcohol (**3d** to **3f**). Alkenes further embedded in the alkyl chain performed well, leading to **3g** and **3h** in high enantioselectivity. An

additional free alcohol did not interfere with catalysis (**3g**), and it was possible to install a challenging nitro-bearing arene (**3i**). When (*Z*)-alkenes were submitted to the same conditions, β -aryl ketone products were isolated in similar yields and enantioselectivity but for the opposite enantiomer of product

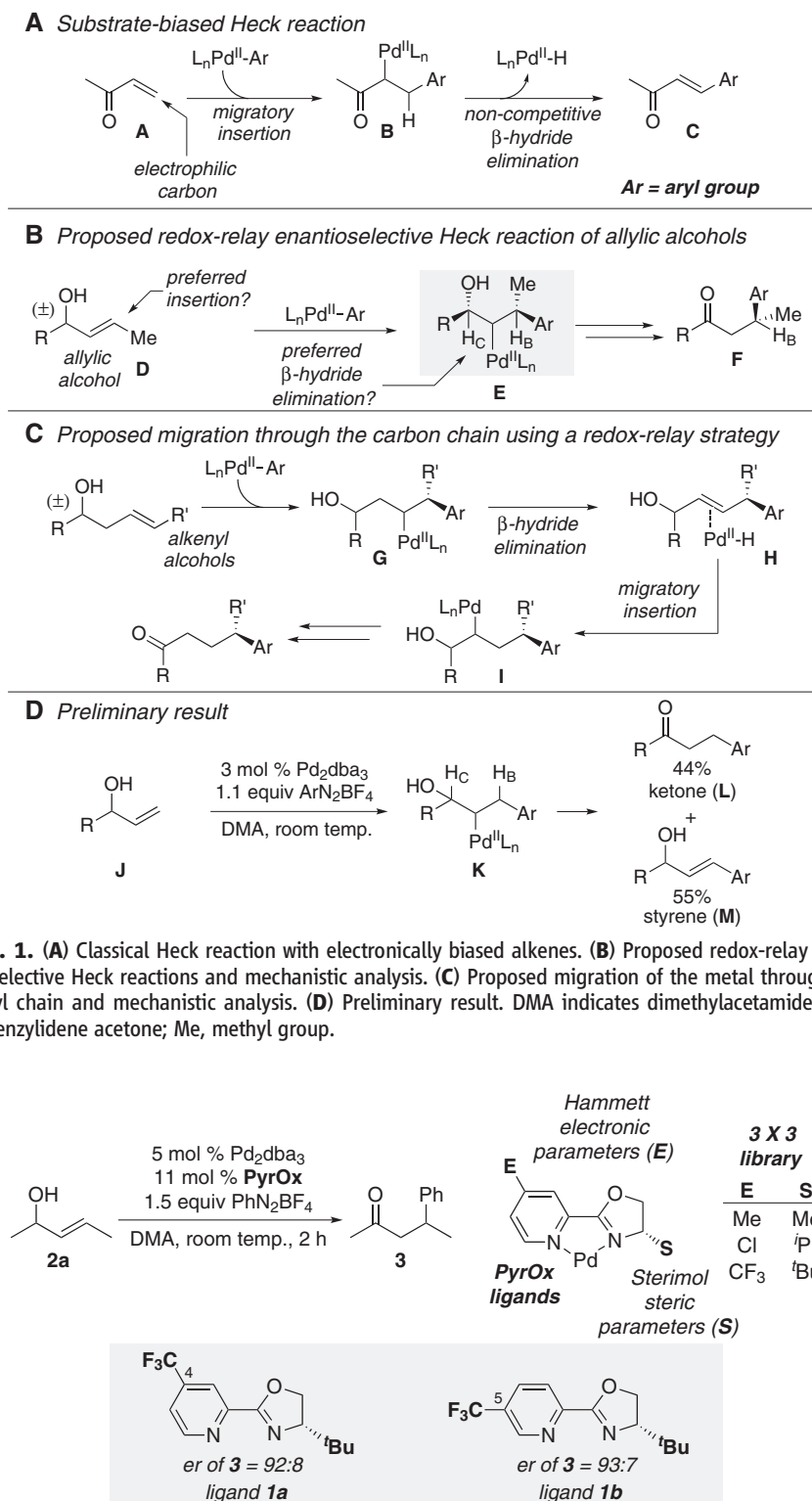


Fig. 2. Ligand selection and optimization protocol of the redox-relay enantioselective Heck reaction of **2a**. Pr, propyl; Bu, butyl group.

(Fig. 3). The yields using (*Z*)-alkenyl substrates tended to be slightly enhanced relative to products delivered from (*E*)-olefins (compare **3i** for each). Again, generally high enantioselectivity was achieved with a diverse array of substrates, including a carbonyl-bearing alkene (**3m**). The best observed result in terms of both enantioselectivity and yield entailed the synthesis of **3j**, wherein an iodide-bearing arene was installed. This product would be difficult to access if aryldiazonium salts were not used. Conveniently accessed racemic substrates are used in all cases above, strongly suggesting catalyst control overriding the chiral nature of the substrate and highlighting the synthetic utility of this transformation.

Whereas many of the β -aryl ketones produced above can in principle be accessed by using various alternative strategies (particularly conjugate addition to α,β -unsaturated carbonyls) (**25**, **30**), γ -aryl carbonyl products are much more difficult to access by using reported asymmetric catalytic methods (**31**, **32**). To effectively form this remote chiral center from a homoallylic alcohol would require selective insertion as well as Pd migration along the carbon chain as discussed above. Therefore, it was gratifying that homoallylic alcohols of various types proved to be excellent substrates for the enantioselective Heck reaction dependent on this redox-relay strategy, leading to generally good yields of γ -aryl ketone

products with excellent enantioselectivity (Fig. 4). Minor amounts of the β -aryl carbonyl products, a result of insertion at the alternative alkenyl carbon, were isolated by using these substrates (<5% in most cases). The scope of the process is not limited to homoallylic secondary alcohols, because aldehydes can be accessed from primary alcohols with electronically diverse aryldiazonium salts in good yields and excellent er values (**4d** to **4h**). As previously described, (*Z*)-alkenes perform modestly better than (*E*)-alkenes in this reaction and again provide the opposite enantiomer of product.

To further evaluate the potential applications of our strategy, we evaluated two substrates wherein the alcohol was an additional carbon away from the

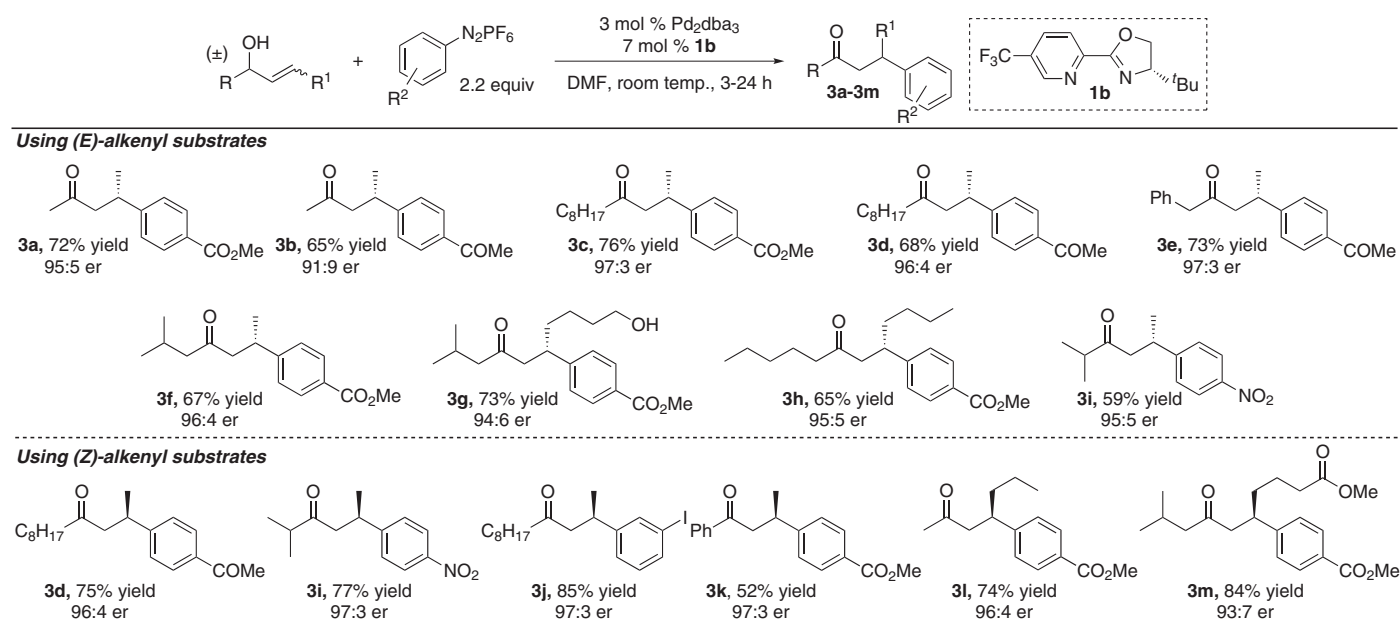
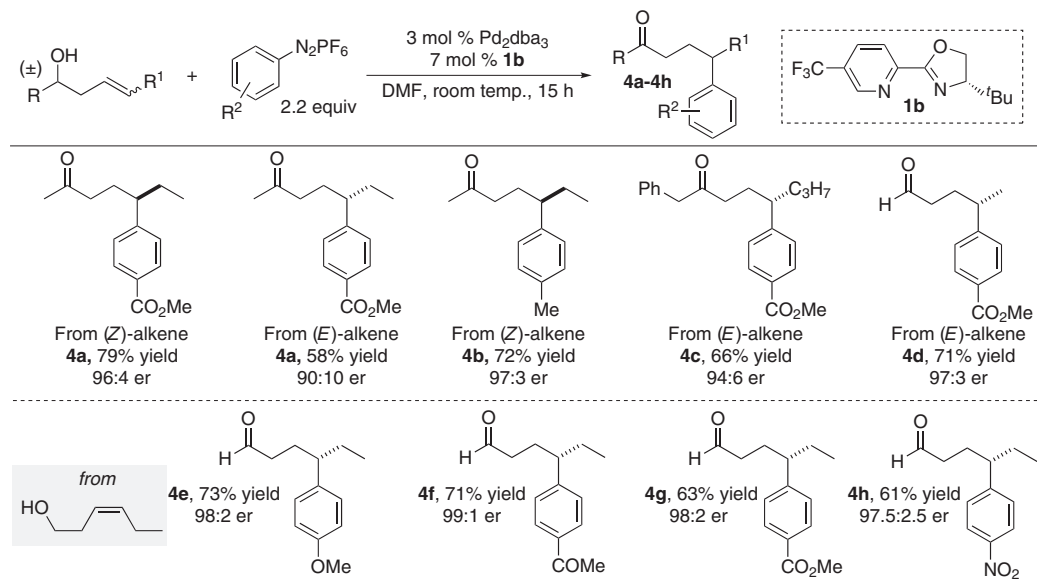


Fig. 3. Scope of the enantioselective redox-relay Heck arylations of racemic allylic alcohols. Absolute configuration was determined by the synthesis of *ar*-(+)-juvabione (**3f**) and comparison to its reported optical rotation data. The balance of the entries was assigned by analogy (**33**, **34**).

Fig. 4. Scope of the enantioselective redox-relay Heck reactions of racemic homoallylic secondary alcohols and primary alcohols.



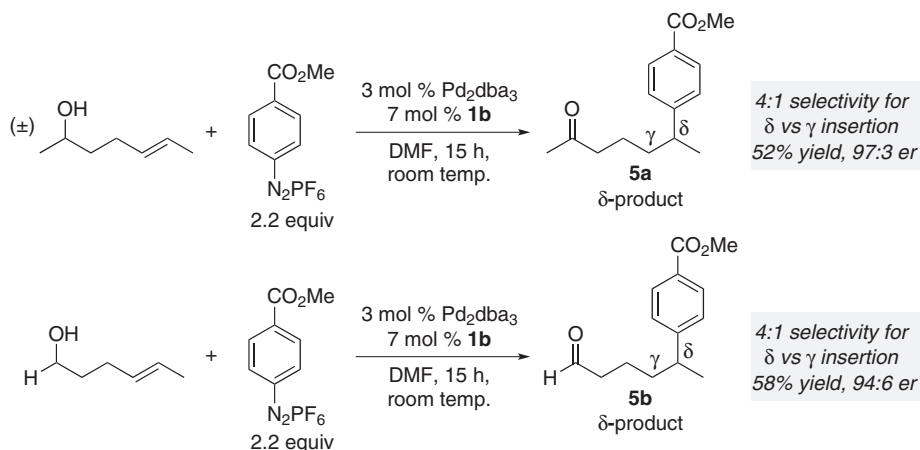


Fig. 5. Evaluation of bis-homoallylic alcohol substrates in the enantioselective Heck reaction.

alkene (Fig. 5). Under the same reaction conditions, the enantioselective Heck reaction successfully resulted in the formation of δ -substituted carbonyl products **5a** and **5b**. The selectivity of insertion is diminished somewhat with ~20% of the γ -substituted product observed. However, high enantioselectivity is still achieved, thus showcasing the robustness and potential power of the catalytic system.

The data presented suggest that the tactic of asymmetrically functionalizing alkenes and transferring unsaturation to a tethered functional group capable of being oxidized can be applied to the synthesis of remote chiral centers. Further studies will be required to establish what features of the catalyst contribute to high selectivity of the insertion events.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/338/6113/1455/DC1
Materials and Methods
Fig. S1
Tables S1 to S5
References (35–51)

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Rapid Folding of DNA into Nanoscale Shapes at Constant Temperature

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We demonstrate that, at constant temperature, hundreds of DNA strands can cooperatively fold a long template DNA strand within minutes into complex nanoscale objects. Folding occurred out of equilibrium along nucleation-driven pathways at temperatures that could be influenced by the choice of sequences, strand lengths, and chain topology. Unfolding occurred in apparent equilibrium at higher temperatures than those for folding. Folding at optimized constant temperatures enabled the rapid production of three-dimensional DNA objects with yields that approached 100%. The results point to similarities with protein folding in spite of chemical and structural differences. The possibility for rapid and high-yield assembly will enable DNA nanotechnology for practical applications.

A candidate route toward the creation of synthetic nanodevices that achieve functionalities such as those of natural protein-based assemblies (1) relies on molecular self-assembly with DNA (2). Design strategies for encoding complex target shapes in DNA se-

quences have flourished (3–15), but the practical assembly of desired objects has often been quite difficult. Low yields and up to week-long reaction times have challenged, in particular, the usefulness of templated three-dimensional (3D) DNA objects in practical applications (16).

In our work, we used results from folding studies with templated DNA objects to find a solution to this problem. “Folding” herein refers to the association of multiple DNA strand species to form a user-defined object through hybridization into double-helical DNA domains, whereas “unfolding” denotes denaturation through strand dissociation. We used real-time fluorometric monitoring and cryogenic reaction quenching to study such folding and unfolding processes as a function of temperature and time (17). From the fluorometric data, we obtained a rate of folding during cooling a solution starting from denaturing temperatures, as well as a rate of unfolding during heating a solution containing folded objects. The rates measured changes in the solution

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content of object-related DNA base pairs at a defined temperature (18). The cryogenic reaction quenching gave complementary insight into the shapes and electrophoretic mobilities of products contained in the reaction mixtures at specified time points and temperatures.

In a first set of experiments, one-pot reaction mixtures containing all required DNA strands were cooled stepwise, starting from denaturing temperatures, in analogy to previously reported protocols (8). We observed a rate of folding that featured a distinct peak at an object-specific temperature and that otherwise fluctuated around zero. Exemplary data obtained for three particular multilayer DNA objects—platelike (19), bricklike (20), and gearlike objects—are shown in Fig. 1, A to C. Results obtained for nontemplated DNA objects (14) are shown in the supplementary text. The data suggested that the plates began to fold only upon cooling to a temperature of 60°C and that their folding was completed at 55°C, whereas the bricks and gears folded within the interval from 55° to 50°C. Cryogenic reaction quenching during stepwise cooling followed by analysis by gel electrophoresis and transmission electron microscopy (TEM) imaging confirmed this interpretation (Fig. 1, D to F): At the high-temperature boundary of the peak in the rate-of-folding data, compacted “molten globule”-like (21) precursors with greater electrophoretic mobility than that of the unfolded species formed in

all reactions (Fig. 1, D to F). Upon further cooling, folded objects appeared and coexisted with the precursors. The conversion to the folded state (as judged by electrophoretic mobility and TEM imaging) was completed at the low-temperature boundary of the peak in the rate-of-folding data.

In a second set of experiments, reaction mixtures containing previously folded DNA objects were heated stepwise. The rate of unfolding featured distinct peaks that were more narrow and shifted toward higher temperatures as compared with the peaks in the rate-of-folding data (Fig. 1, A to C). These data suggested that the objects remained folded up to higher temperatures compared with the temperatures at which they previously formed; this result was confirmed by cryogenic reaction quenching (Fig. 1, D to F). Because cooling and heating gave different results, the data pointed to nonequilibrium processes. Experiments in which folded objects were incubated for either 2 hours or 2 days at set temperatures within the peak in the rate-of-unfolding data gave unchanged results regarding the temperatures at which the objects unfolded (fig. S4B). By contrast, experiments in which unfolded objects were incubated for 2 days at set temperatures within the peak range in the rate-of-folding data led to folding at higher temperatures as opposed to 2-hour-long incubation (fig. S4A). Thus, folding rather than unfolding was not in equilibrium at the time scales that we tested.

The data also revealed strong cooperativity in folding and unfolding. For independent hybridization of full-length DNA strands to the template strand, base pairs are expected to form over a broad temperature range ($\Delta T > 30^\circ\text{C}$) at temperatures well above 60°C. By contrast, for independent hybridization of all double-stranded DNA (dsDNA) domains designed in each object, base pairs are expected to also form over a broad temperature range ($\Delta T > 30^\circ\text{C}$), but mostly at temperatures well below 45°C. Both cases were not observed; instead, all objects that we tested folded at intermediate temperatures between 45° and 60°C, and base pairs formed only within narrow temperature intervals ($\Delta T \sim 4^\circ\text{C}$) (Fig. 1). Analogous reasoning applied to the observed cooperativity during unfolding.

In a third set of experiments, we studied the folding and unfolding of a hinged-bar-shaped object consisting of two independent domains organized on interleaved template strand segments. We observed domain-wide cooperative folding and unfolding, similar to in proteins (22, 23): The two domains folded independently in two distinct folding transitions (Fig. 2A). TEM imaging of reaction products shock-frozen at the temperature between the two peaks in the rate-of-folding data revealed objects in which the longer “arm” domain was folded and an unstructured loop was hanging off, whereas reaction mixtures extracted at the lower-temperature boundary of both peaks contained fully folded hinged-bar objects (Fig. 2B). The unfolding of the hinged-bar

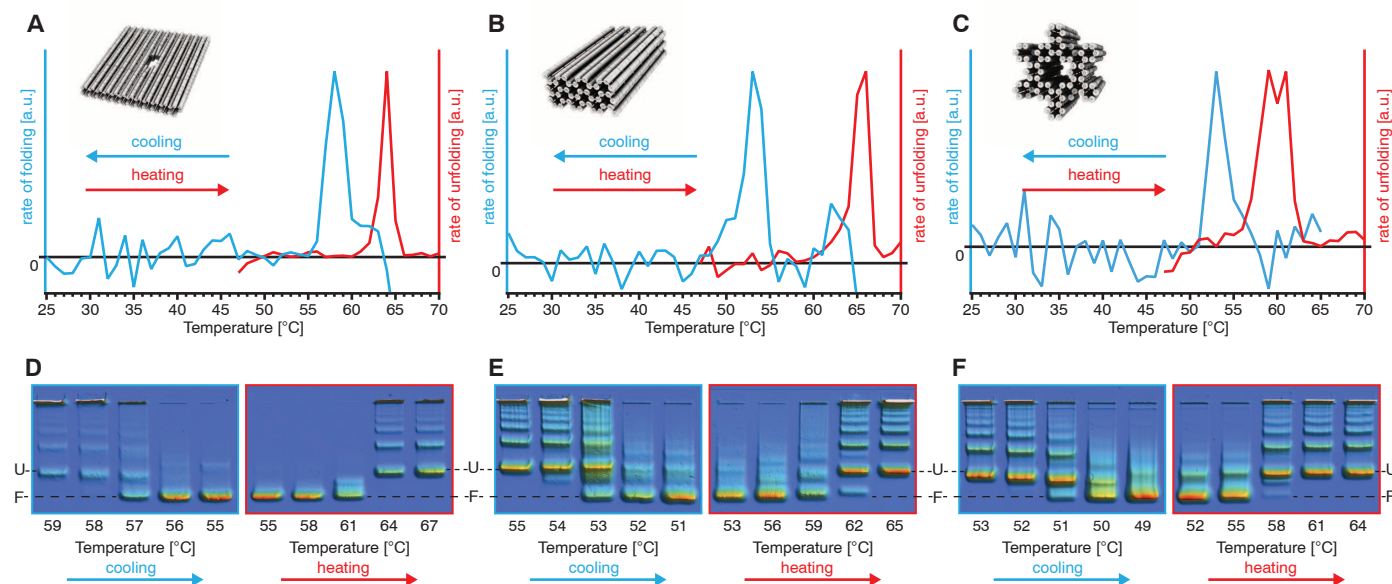


Fig. 1. Folding and unfolding of templated multilayer DNA objects as a function of temperature. (A) Fluorometrically obtained rates of folding (blue line) and unfolding (red line) for a templated multilayer platelike DNA object (inset). For folding, after a fluorescence equilibration period (18), one-pot reaction mixtures were denatured for 5 min at 65°C and then cooled stepwise to room temperature. The rate of cooling was 1 K per 15 min from 64° to 60°C and 1 K per 3 hours from 60° to 25°C. For unfolding, unpurified one-pot reaction mixtures containing previously folded objects were heated stepwise with a rate of 1 K per 3 hours. a.u., arbitrary units. (B and C) Same as in (A), but for templated bricklike and gearlike objects, respectively. (D to F) Agarose gel-electrophoretic mobility analysis of reaction products in temperature-resolved cryogenic shock-freezing

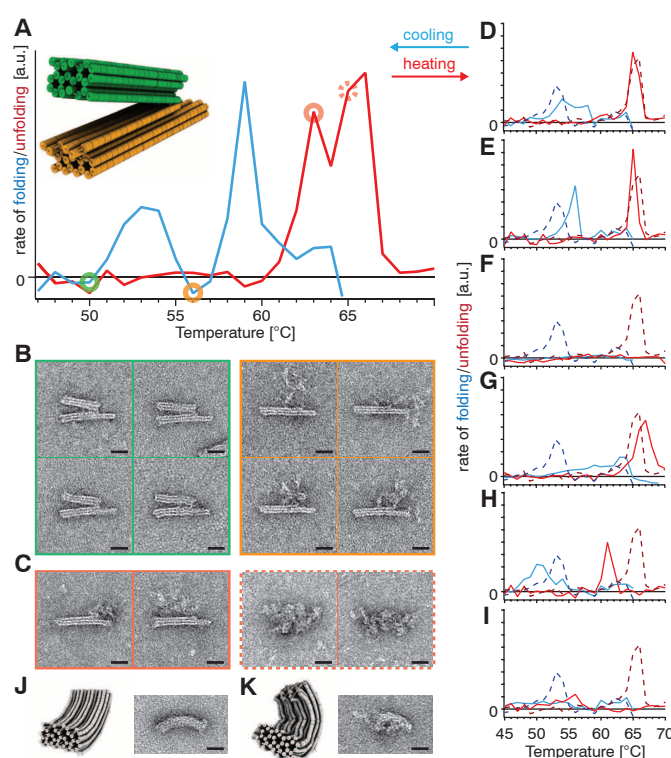
experiments. (Left) One-pot reaction mixtures were subjected to stepwise cooling as in (A) to (C), but samples were extracted and shock-frozen in liquid nitrogen after the incubation step at the indicated temperatures was completed. (Right) Reaction mixtures containing folded objects were incubated for 2 hours at the temperatures indicated, then extracted and shock-frozen. U and F denote bands corresponding to unfolded and folded species, respectively. Bands in between the U and F markers correspond to compacted precursors (folding) or partially unfolded objects (unfolding). Gel photographs were depicted in 3D landscape mode to support the comparison of band intensities. Source data in grayscale are given in fig. S5. See figs. S9 to S12 for additional TEM data and figs. S19 to S21 for design details. Sequences are given in tables S1 to S3.

object upon heating also occurred in two steps: First the short arm of the object unfolded, followed by unfolding of the long arm (Fig. 2C).

In a fourth set of experiments, we explored the influence of design details on the folding and unfolding transitions of the same overall shape (a bricklike object). Assuming that nuclei for folding consist of the dsDNA domains with the highest independent thermal stability as defined by design, template sequence permutations will influence the pathways available for nonequilibrium folding and, thus, affect the temperatures at which folding occurs. By contrast, for near-equilibrium unfolding, the temperatures at which sequence variants of an object unfold should be similar, because the total number and composition of base pairs will be identical. We observed this behavior for sequence variants of the bricklike object (Fig. 2, D and E). Single-nucleotide mismatches in all dsDNA domains defined in the bricklike object led to failure to fold, as reflected in a missing peak in the fluorometric rate-of-folding trace (Fig. 2F). Increasing the average length of staple strands from 42 to 100 bases led to a broadening and shifting toward higher temperatures of the peak in the rate of folding, but had little influence on the rate of unfolding (Fig. 2G). Additional energetic penalties—for instance, bending (9)—are expected to lead to destabilization and, thus, to a shift of folding and unfolding transitions to lower temperatures. We observed this result for a moderately bent version of the bricklike object (Fig. 2, H and J). A strongly bent version did not fold anymore, as seen in TEM data and in the loss of a distinct peak in the rate-of-folding trace (Fig. 2, I and K). Finally, varying the staple-strand routing while using identical template-strand routing and sequence permutation influenced both folding and unfolding transitions of the bricklike object in various ways (fig. S7). Thus, design choices shifted folding and unfolding temperatures both together and independently for the same shape, which may allow for rationally tuning DNA objects to assemble and disassemble at target temperatures.

Given the high degree of cooperativity and the sharply defined transition temperatures for folding templated DNA objects, we hypothesized that such objects might be capable of folding at constant temperature, thus dispensing with annealing (3–15, 24). A fifth set of experiments revealed that this was true (Fig. 3 and fig. S8). At the low-temperature boundary T_{fold} of the peak in the object-specific, rate-of-folding data, the process of folding occurred on a time scale of minutes. Deviating from this optimal T_{fold} slowed down or inhibited folding entirely (Fig. 3A and fig. S8, A and G). Folded DNA plates, bricks, and gears appeared in the reactions after 15, 30, and 40 min, respectively, of incubation at constant T_{fold} , as seen in time-resolved cryogenic reaction-quench experiments (Fig. 3C and fig. S8, C and G). Note that prior thermal denaturation by a heat shock (e.g., 15 min at 65°C), presumably to resolve secondary structures in the template strand, was obligatory to achieve folding. As in the stepwise cooling experi-

Fig. 2. Domain-wide folding cooperativity and the influence of design details on folding and unfolding temperatures. (A) Fluorometric rate of folding (blue line) upon cooling and rate of unfolding (red line) upon heating obtained for a templated DNA object shaped like a hinged bar. (B) Typical TEM micrographs of particles contained in hinged-bar reaction mixtures upon cooling, as in (A), that were shock-frozen at 56°C (right) and at 50°C (left). Scale bars, 20 nm. (C) Same as in (B), but from reheating folded objects and shock-freezing at 63°C (left) and at 65°C (right). Scale bars, 20 nm. (D to G) Fluorometric rate of folding (solid blue lines) upon stepwise cooling and the rate of unfolding (solid red lines) upon stepwise reheating of the formerly cooled reaction mixtures, as obtained for design variants of the bricklike object from Fig. 1. Results for the original version are given as dashed lines. (D and E) Sequence permutations. (F) Single-nucleotide mismatches in all double-helical DNA domains. (G) Average staple-strand length: 100 bases instead of 42. (H and I) Same as in (D) to (G), but for bent bricklike objects. (J) Typical TEM micrograph of a folded bricklike object from (H) with a moderate bend. Scale bar, 20 nm. (K) Same as in (J), but for a bricklike object designed with strong bend, which failed to fold. Scale bar, 20 nm. See figs. S13 and S14 for additional TEM data and figs. S22 to S27 for design details. Sequences are given in tables S4 to S16.



ments in Fig. 1, the objects folded across compacted precursors at constant T_{fold} . Unfolding at the temperature of the peak in the rate-of-unfolding data occurred rapidly within seconds (Fig. 3D and fig. S8, D and H). Time-resolved fluorometric data indicated reaction completion at comparable time scales, as seen for folding and unfolding in the cryogenic experiments (Fig. 3, E and F). Thus, the time scales for folding may also be determined using the more convenient, parallelizable fluorometric assay. A templated single-layer DNA object folded at 53°C within 5 min (Fig. 3, G to J). Finally, constant-temperature folding at T_{fold} strongly enhanced the yield of correctly folded particles (Fig. 3, K and L) by avoiding side-product formation and reducing material loss through thermal degradation. When reaction quality was measured electrophoretically by the ratio of leading band intensity (reflecting desired monomeric folded objects) versus aggregate band intensity, a 330-fold improvement over previous protocols (8) could be achieved for the gearlike object (Fig. 3K) (215-fold and sevenfold improvements for the DNA brick and plate objects, respectively). The absolute yield approached 100% when we compared folded particles with total particles that were seen in TEM images of excess-staple-free, but not otherwise purified, reaction products (Fig. 3L and figs. S15 to S18). Note that these results were achieved in

reactions that lasted 1% of the duration of previous protocols for making such objects (8).

The possibility for rapid, high-yield production of complex DNA objects will support achieving usefulness of DNA nanotechnology in practical applications. Fine-tuning of DNA devices through serial cycles of functional selection (25) becomes practically feasible, and prospects for the hierarchical assembly (8, 26–28) of larger objects are opened, where a high yield of well-folded building blocks is one important prerequisite. Furthermore, the finding that complex DNA objects are capable of folding at tunable constant temperatures within the lifetime of a cell suggests that assembly of such objects in cell culture or even within a cell may be possible.

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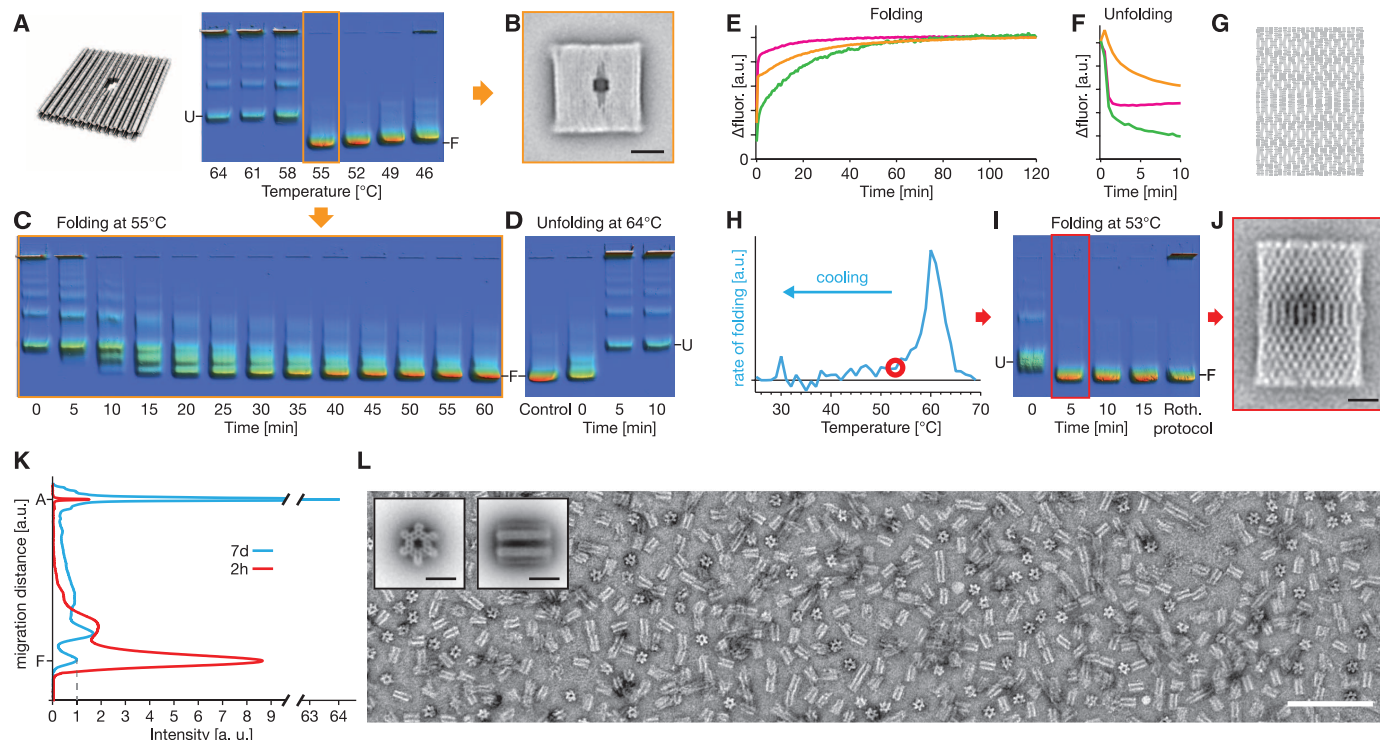


Fig. 3. Time-resolved folding and unfolding of templated DNA objects at constant temperature. (A) Agarose gel mobility analysis of products in reaction mixtures for the platelike object that were incubated for 2 hours at the temperatures indicated. (B) Averaged single-particle TEM micrograph obtained from excess-staple-free but not otherwise purified products in the boxed reaction in (A). Scale bar, 20 nm. See fig. S9 for additional TEM data. (C) Time-resolved folding of the platelike object at constant 55°C, as obtained through shock-freezing followed by agarose gel electrophoresis. (D) Time-resolved unfolding at constant 64°C. (E) Temporal evolution of the normalized SG fluorescence intensity in full assembly reaction mixtures minus intensity recorded in staple-free only assembly reaction mixtures for DNA plates (orange) at 55°C, bricks (green) at 50°C, and gears (magenta) at 49°C. (F) Same as in (E), but for unfolding at the temperatures indicated in (D), fig. S8D, and fig. S8H, respectively. (G) Model of a templated 2D DNA rectangle [adapted from (4)]. (H) Rate of folding obtained

upon stepwise cooling of the rectangle. (I) Time-resolved folding as in (C) and fig. S8, C and G, at 53°C of the rectangle object after prior denaturation at 95°C for 1 min. Roth. protocol denotes the lane on which products obtained using a previous protocol (4) were electrophoresed. Note the absence of aggregates in the boxed lane. (J) Averaged TEM micrograph of particles observed in the boxed reaction in (I). Scale bar, 20 nm. Note the correspondence to (G). (K) Comparison of gel-electrophoretic lane intensity profiles obtained for products from 2-hour constant-temperature folding (red line) versus annealing as in (8) (blue line) for the gearlike object. "A" denotes aggregates stuck in the gel pocket; "F" indicates folded objects. (L) Field-of-view negative-stain TEM micrograph of unpurified DNA gear reaction products (stripped from excess staple strands by filtration, but not otherwise purified). Black scale bars, 20 nm; white scale bar, 200 nm. See fig. S8 for an analysis, as in (A) to (D), for the bricklike and gearlike objects. Also see figs. S14 to S18 for field-of-view TEM data for plate-, brick-, and gearlike objects.

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- In the fluorometric experiments, trace amounts of the organic dye SYBR green (SG) were added to one-pot self-assembly reaction mixtures (18). The use of low concentrations of SG corresponding to one SG molecule per ~900 possible DNA base pairs in solution ensured negligible perturbation of the DNA hybridization processes. The fluorescence brightness of SG is enhanced upon intercalation into dsDNA domains (29); recording the SG fluorescence intensity can thus provide a measure for the evolution of the overall content of DNA base pairs in solution. A specific signal for the formation of object-related DNA base pairs upon incubation for time t at temperature T is obtained by comparison with reference reactions (18). For templated DNA objects—i.e., objects that form by staple DNA strand-assisted folding of a much longer scaffold DNA template molecule (4)—reference reactions were prepared that included the respective set of staple DNA oligonucleotides but lacked the scaffold DNA strand. Cryogenic shock-freezing of a reaction mixture in liquid

- nitrogen quenched folding and unfolding processes and preserved the state of the reaction at the time and temperature of freezing (18). After quick thawing, the solution content was analyzed using agarose gel electrophoresis and/or direct imaging with negative-stain TEM. Gel electrophoresis and TEM imaging were performed as previously described (30). Excess staple strands were removed by molecular weight cut-off filtration with 100-kD spin filters. Objects were designed using caDNAo (31); models were computed using CanDo (30, 32).
- Materials and methods are available as supplementary materials on Science Online.
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Supplementary Materials
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 Materials and Methods
 Supplementary Text
 Figs. S1 to S34
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Pheromonal Induction of Spatial Learning in Mice

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Many mammals use scent marking for sexual and competitive advertisement, but little is known about the mechanism by which scents are used to locate mates and competitors. We show that darcin, an involatile protein sex pheromone in male mouse urine, can rapidly condition preference for its remembered location among females and competitor males so that animals prefer to spend time in the site even when scent is absent. Learned spatial preference is conditioned through contact with darcin in a single trial and remembered for approximately 14 days. This pheromone-induced learning allows animals to relocate sites of particular social relevance and provides proof that pheromones such as darcin can be highly potent stimuli for social learning.

Scent marks deposited in the environment are used widely by mammals and other vertebrates to advertise location, identity, and status to other conspecifics (1). Males in particular invest heavily in territorial scent marks and countermarks to advertise their competitive ability (2, 3). These scent marks are important for female preference between males and for regulating interactions between competitors (2, 4, 5). However, surprisingly little is known about how scent marks attract conspecifics to particular sites and to the scent owner. It is assumed that this involves active detection and orientation toward odor molecules emanating from the scent source (6). At its simplest, animals may detect a plume of air- or water-borne odor molecules and orient along a concentration gradient toward the source

(6) or follow trail pheromones left on the substrate, detected at a much closer distance (7–9). The role of learning and spatial memory in scent mark communication has received considerably less attention. We hypothesized that learning stimulated by specific pheromones is an essential component of the response to scent marks that are left in static locations to advertise use of the site by a particular scent owner.

Studies examining the rewarding properties of sexual experience in rodents demonstrate that multiple daily encounters with the opposite sex in one specific location (10, 11), or just with attractive scents from the opposite sex (11–13), can induce remembered preference for the location itself through associative learning. However, the stimuli in scent marks that induce spatial learning and the rapidity of learning have not been examined. We used the attraction of female house mice to urine scent marks that male mice deposit throughout their defended territory (14, 15) to determine whether specific pheromones may play a role. Outbred wild-stock house mice were used to ensure that both signal and response reflect

natural behavior across different genotypes (16). Because female laboratory mice demonstrate a conditioned place preference after several daily encounters with cage bedding soiled by males (13), we first tested whether this is specifically conditioned by urine that males use for territorial marking and whether repeated encounter is required for learning. Females were given two small Petri dishes placed in opposite halves of a clean test arena, sited on different textured floor tiles as spatial cues. During 10-min daily learning sessions, one dish contained male urine (50 μ L) and the other a water control. Conditioned place preference (CPP) was tested with no urine present 24 hours after the last learning session. Females spent more time in the urine dish than in the control dish over three daily learning sessions and developed a CPP for the remembered location when tested 24 hours later (Fig. 1A). This confirmed that the scent that conditions female place preference is in male mouse urine. Repeated exposure was not necessary to induce CPP, which was as strong after three, two, or only one brief daily exposure to the location of male urine (Kruskal-Wallis $\chi^2 = 0.71$, 2 df, $P = 0.70$) (Fig. 1, A to C). Even after only a single learning session, females spent approximately five times longer in the remembered location of male urine as compared with the control, showing as much bias as when urine was present. Learned preference occurred after just 13.6 ± 3.0 s of close sniffing at the male urine stimulus 24 hours earlier. In contrast, females spent relatively little time near equivalent urine from an unfamiliar female and showed no conditioned preference for its location (Fig. 1D), responses that differed from attraction to male urine location [learning day: matched-pair t test (t_{21}) = 2.37, $P = 0.028$; CPP: $t_{21} = 3.24$, $P = 0.004$].

Place preference for male scent location was remembered for a surprisingly long period. Pref-

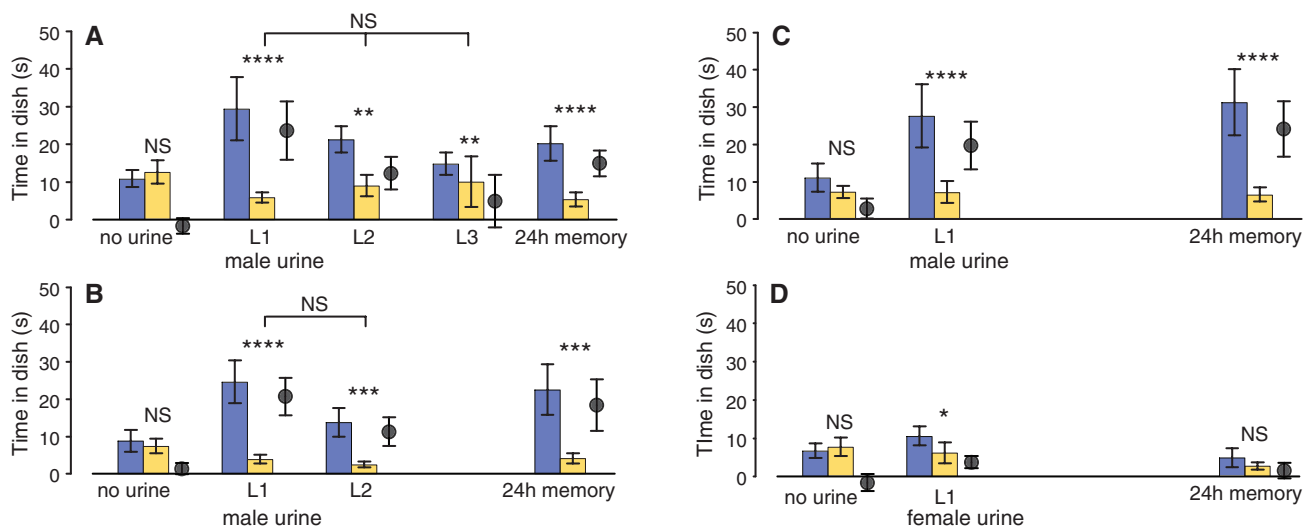


Fig. 1. Female sexual attraction to male urine scent marks and conditioned place preference. After confirming no side bias (no urine), female mice were given test urine versus water in two dishes in 10-min daily learning sessions (L1 to L3). CPP was tested 24 hours later with no urine present (24-hour memory). Females were given (A, B, and C) unfamiliar male or (D) female urine for three (A) $n = 10$ subjects, two

(B) $n = 12$ subjects] or one [(C) and (D) $n = 12$ subjects] learning sessions. Greater time spent in the urine (blue bars) versus control dish (yellow bars) was assessed using one-tailed paired t tests (data log transformed to meet parametric assumptions): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$. Circles show matched-pair difference in time spent in test minus control dish. Data are means $\pm$ SEM.

erence was evident when the interval between a single 10-min scent exposure and test of CPP was 14 days, although no CPP was retained after an interval of 28 days (Fig. 2A and fig. S1). However, extinction occurred rapidly once animals encountered no scent in a previously conditioned site: When tested on two successive days with no scent present, CPP was absent on the repeated test day (Fig. 2, B and C). Revisiting the remembered site between tests itself did not disrupt preference if male scent was still present (Fig. 1B). Thus, females form a memory of male scent mark location through associative learning on a single encounter that can be remembered for at least 14 days; however, they rapidly update remembered spatial associations if male scent is

absent when revisiting a site. Mice are most likely to be using physical cues (floor tiles with different surface textures) and visual landmarks (overhead red light source) to remember scent location, although they could use acoustic cues (supplementary materials, materials and methods).

Associative learning of scent location could be a general response to male-conspecific odors. Many species-specific androgen-dependent volatile components in adult male mouse urine (17–19) could allow females to detect male scent marks. However, when nasal contact with urine was prevented by use of a mesh screen, airborne volatiles failed to condition any preference for scent location (fig. S2A). Airborne odor was sniffed during learning ($t_{11} = 2.00$, $P = 0.039$),

but females spent no more time near this than near water, which is consistent with previous findings that male airborne volatiles do not stimulate instinctive sexual attraction in mice (14, 20). Instinctive sexual attraction is elicited by one specific involatile sex pheromone expressed by adult males: a major urinary protein (MUP) named darcin (MGI:3651981, *Mup20*) that is detected on nasal contact (15). To test our hypothesis that darcin conditions females to show the same preference toward associated spatial cues, we expressed darcin as a recombinant protein (r-darcin) in *Escherichia coli* along with two other control MUPs: r-MUP7 (MGI:3709615) and r-MUP11 (MGI:3709617). Spatial conditioning was induced only by darcin, whether presented alone or added

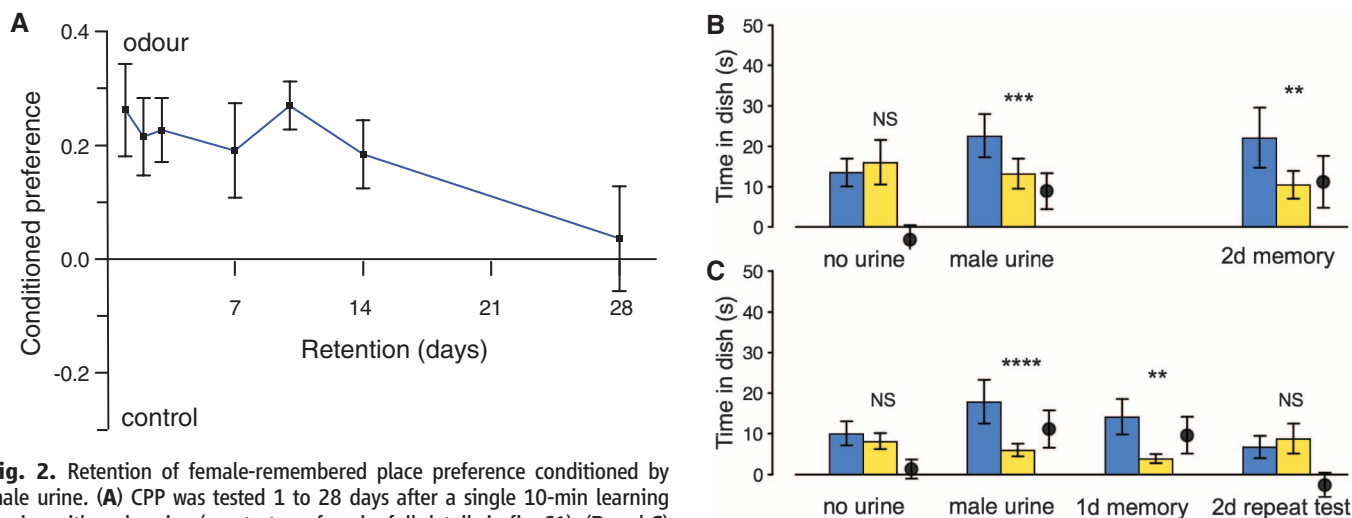
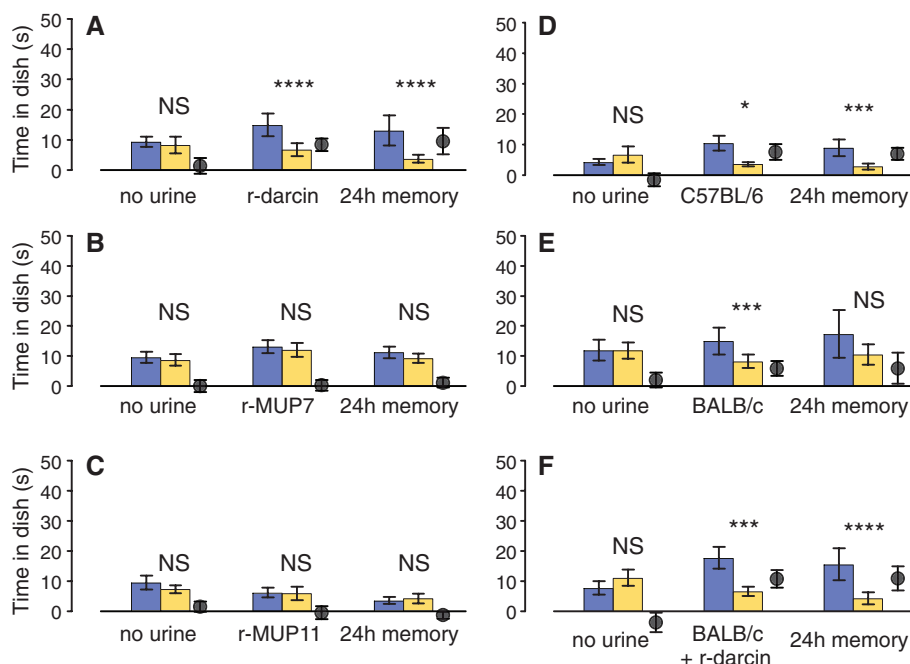


Fig. 2. Retention of female-remembered place preference conditioned by male urine. (A) CPP was tested 1 to 28 days after a single 10-min learning session with male urine (one test per female; full details in fig. S1). (B and C) CPP was tested 2 days after the learning session with no intervening exposure to the test arena [(B) $n = 11$ subjects], or both 1 day and 2 days after learning [(C) $n = 20$ subjects]. Key and statistical tests are as in Fig. 1.

Fig. 3. Darcin stimulates female conditioned preference for male urine location. CPP was assessed 24 hours after a single 10-min learning session with test stimulus versus (A to C and F) control buffer or (D and E) water. (A) r-darcin (1 $\mu\text{g}/\mu\text{l}$ buffer, $n = 18$ subjects); (B) r-MUP7 (1 $\mu\text{g}/\mu\text{l}$ buffer, $n = 37$ subjects) (supplementary materials, materials and methods); (C) r-MUP11 (1 $\mu\text{g}/\mu\text{l}$ buffer, $n = 18$ subjects); (D) C57BL/6 male urine containing 8 $\mu\text{g}/\mu\text{l}$ protein, including 1 $\mu\text{g}/\mu\text{l}$ natural darcin ($n = 10$ subjects); (E) BALB/c male urine containing $<0.1 \mu\text{g}/\mu\text{l}$ darcin ($n = 11$ subjects); (F) BALB/c male urine plus r-darcin (1 $\mu\text{g}/\mu\text{l}$, $n = 12$ subjects). Greater time in test dish was assessed by Wilcoxon [(A) to (C)] or t tests (data log transformed). Key is as in Fig. 1.



to male urine. Females spent more time with r-darcin than with a buffer control when present and showed a CPP that was just as strong 24 hours later (Fig. 3A). Female response to r-darcin alone was as strong as that toward intact male urine containing the same amount of darcin (inbred laboratory strain C57BL/6) (Fig. 3D). The lack of attraction to other r-MUPs (Fig. 3, B and C) differed from r-darcin both for learning ($\chi^2 = 9.03$, 2 df, $P = 0.011$) and CPP ($\chi^2 = 9.78$, 2 df, $P = 0.008$). Further, females showed no attraction

or CPP for inbred BALB/c male urine (Fig. 3E), which has naturally high levels of MUP7 and MUP11 (21) but lacks normal adult male expression of darcin (15), unless r-darcin was added (Fig. 3F).

Darcin not only reliably conditions spatial preference, it also induces female learned attraction to the airborne urinary odor of the male scent owner (15). When given prior contact with male urine containing darcin, females learned an attraction to airborne urinary volatiles from this familiar

urine but not toward unfamiliar urinary volatiles (fig. S2). However, there was no second-order conditioning, by which contact with darcin conditions attraction to airborne volatiles and the attractive airborne volatiles then condition remembered preference for airborne scent location, even after multiple learning sessions (fig. S2, B and C). Only direct contact with darcin itself conditions a remembered preference for male scent location.

Male scent marks advertise to females but also convey information and a competitive signal to other males. Males are strongly motivated to monitor and countermark signals from potential competitors, particularly those within a competitive male's own scent-marked territory (2, 5). We tested whether male mice remember the location of another male's scent marks and whether this is induced by darcin. Singly housed adult males (representing competitive individual territory owners) spent time near unfamiliar male urine as expected and showed a strong conditioned preference for this location 24 hours later (Fig. 4A). This CPP was evident, if weaker, 14 days after urine encounter (Fig. 4B). Males spent time near airborne urinary volatiles from an unfamiliar male when nasal contact was prevented (unlike females), but there was no CPP for the airborne scent location (Fig. 4C). Exactly as in females, place preference was conditioned only through contact with darcin, which elicited a CPP even when presented alone (Fig. 4D). The lack of response to other r-MUPs (Fig. 4, E and F) differed significantly from the response to r-darcin on both learning day ($\chi^2 = 7.09$, 2 df, $P = 0.029$) and CPP test ($\chi^2 = 9.59$, 2 df, $P = 0.008$). BALB/c male urine without darcin failed to condition place preference (Fig. 4G) unless this male sex pheromone was added (Fig. 4H). Even when presented alone, preference for the location of r-darcin was remembered for 14 days by both sexes (z score = -2.57 , $P = 0.004$; effect of sex, Mann-Whitney z score = -0.87 , $P = 0.41$), which confirms that darcin is as potent as intact male urine in stimulating prolonged memory of male scent location.

Male mice express darcin themselves but spent very little time near their own urine during learning trials (Fig. 4, I and J)—substantially less than near unfamiliar male urine (Mann-Whitney z score = -3.78 , $P < 0.0005$). Although own urine elicited a very weak CPP (Fig. 4I), this was much weaker than that conditioned by urine from another male (z score = -2.92 , $P = 0.004$) (Fig. 4A). Thus, individual scent “signatures” in urine allow males to recognize own urine quickly, reducing contact with darcin (3.7 ± 0.6 s of close-contact sniffing versus 16.5 ± 1.5 s toward unfamiliar male urine) and minimizing CPP to own scent marks.

We have discovered a new mechanism of spatial learning induced by a specific pheromone that underlies the ability of animals to relocate and spend time at sites where they have previously encountered male scent. Darcin induces single-trial learning of place preference that is

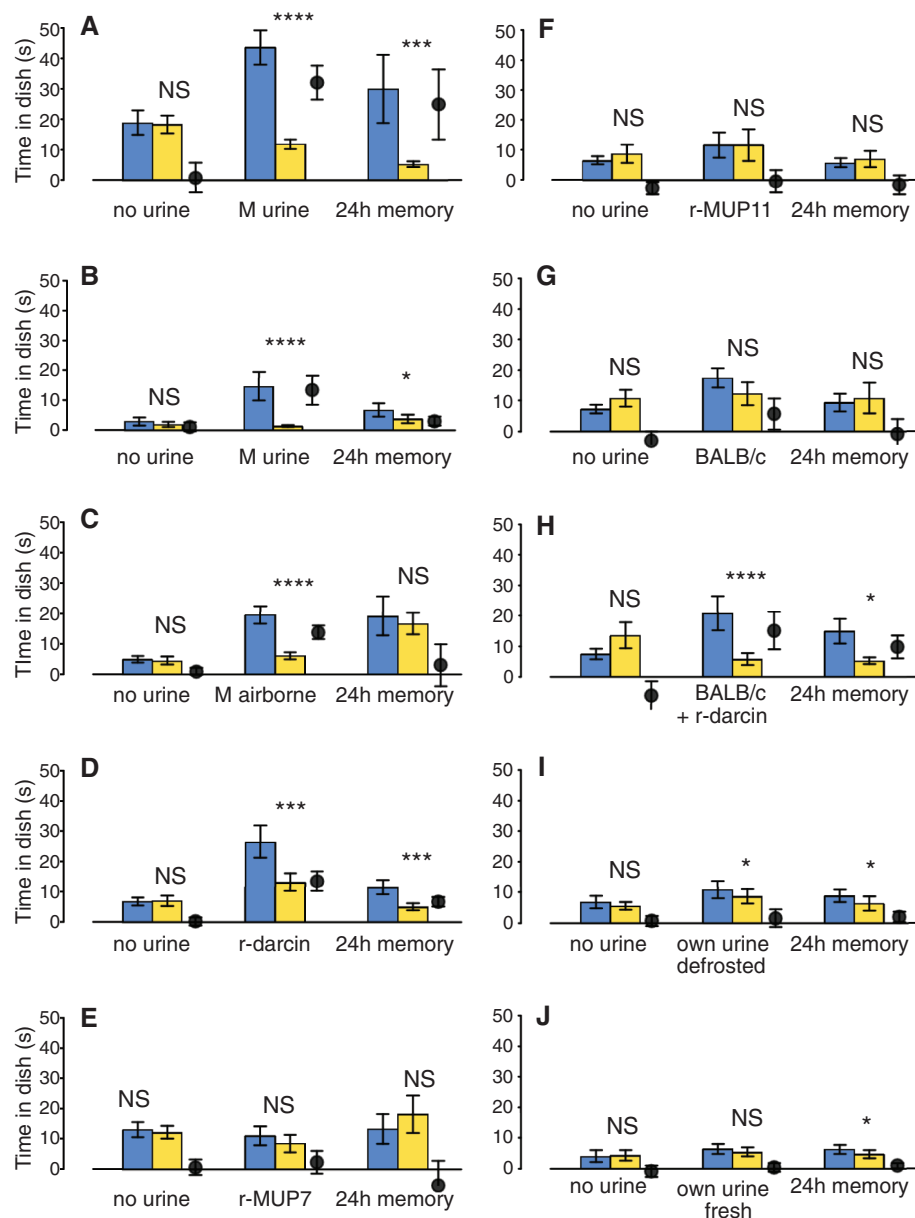


Fig. 4. Darcin stimulates conditioned preference for male urine location among competitor males. CPP was assessed 24 hours or 14 days after a single 10-min learning session with test stimulus versus (A to C, G, I, and J) control water or (D to F and H) buffer. Unfamiliar wild-stock male urine (A) 24 hours or (B) 14 days after contact, or (C) 24 hours after exposure to airborne urinary volatiles; (D) r-darcin (1 μ g/ μ l buffer); (E) r-MUP7 (1 μ g/ μ l buffer); (F) r-MUP11 (1 μ g/ μ l buffer); (G) BALB/c male urine containing <0.1 μ g/ μ l darcin; (H) BALB/c male urine plus r-darcin (1 μ g/ μ l); $n = 12$ wild-stock males tested for each. Own urine (I) frozen prior to testing ($n = 20$ subjects) or (J) collected immediately before learning session ($n = 11$ subjects). Greater time in test dish was assessed by Wilcoxon [(D) to (H)] or t tests (data log transformed). Key is as in Fig. 1.

remembered for ~2 weeks, although extinction is rapid once animals learn that the involatile pheromone is no longer present. This suggests that darcin is a particularly salient social cue for attracting mice of both sexes. It appears to activate a specific mechanism of associative learning so that instinctive attraction to spend time near this pheromone is extended both to its learned location and to airborne odors associated with the pheromone (15). Single-trial learning of associated odors is induced by another pheromone from rabbit mammary glands to improve pup ability to localize nipples efficiently (22), but spatial learning is unlikely to be involved.

This establishes a new role for mammalian pheromones in stimulating learned as well as instinctive social responses. Pheromone-induced learning may be much more important than previously recognized, allowing animals to remember and rapidly relocate scent-marked sites of particular social relevance and driving the flexible individual-specific social responses that typify mammals. Even though all adult male mice produce the same sex pheromone, pheromone-induced learning strongly reinforces attraction to a particular individual male and his location. Learned attraction to the individual-specific airborne odor associated with darcin further targets attraction to other scent marks emitting the same individual's odor, resulting in contact with darcin and conditioned preference for other scent-marked

sites as well as to the individual male himself. Thus, pheromone-induced learning reinforces attraction to a particular male much more effectively than does simple attraction to the pheromone alone. The reliable and rapid learning induced by darcin among both female and male mice provides a valuable and tractable new model to investigate the neural pathways and mechanisms involved in spatial learning and in the learning of complex individual-specific social odors in response to a specific pheromone stimulus. It may also help to establish how such social information about individual conspecifics is stored and integrated in the brain.

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Supplementary Materials

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Materials and Methods
Figs. S1 and S2
References (23–27)
Data File S1

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EZH2 Oncogenic Activity in Castration-Resistant Prostate Cancer Cells Is Polycomb-Independent

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Epigenetic regulators represent a promising new class of therapeutic targets for cancer. Enhancer of zeste homolog 2 (EZH2), a subunit of Polycomb repressive complex 2 (PRC2), silences gene expression via its histone methyltransferase activity. We found that the oncogenic function of EZH2 in cells of castration-resistant prostate cancer is independent of its role as a transcriptional repressor. Instead, it involves the ability of EZH2 to act as a coactivator for critical transcription factors including the androgen receptor. This functional switch is dependent on phosphorylation of EZH2 and requires an intact methyltransferase domain. Hence, targeting the non-PRC2 function of EZH2 may have therapeutic efficacy for treating metastatic, hormone-refractory prostate cancer.

Factors involved in maintaining the epigenetic state of the cell are frequently altered in cancer and are promising therapeutic targets. The expression of EZH2 (enhancer of zeste homolog 2) is correlated with prostate cancer progression, especially to its lethal castration-resistant state (CRPC) (1). EZH2 is the catalytic subunit of Polycomb repressive complex 2 (PRC2), which

silences transcription through trimethylation of Lys²⁷ on histone H3 (H3K27me3) (2). Most studies have focused on PRC2-mediated repression as the oncogenic mechanism of EZH2. In addition, tumor suppressors such as *DAB2IP* have been reported as EZH2 or PRC2 targets (3). However, substantial studies have indicated that both *Drosophila* E(z) (enhancer of zeste) and EZH2 have

potential functions other than that of a transcriptional repressor (4–6), although the mechanisms are unclear.

We used the LNCaP cell line as a model of androgen-dependent prostate cancer and LNCaP-abl (abl), its androgen-independent derivative (7), to study EZH2 function in the progression of prostate cancer to CRPC. As is the case for clinical tumors (1), EZH2 levels in abl cells were much higher than in LNCaP cells (Fig. 1A). EZH2 silencing had a more profound effect on the androgen-independent growth of abl cells than on the androgen-dependent growth of LNCaP cells (Fig. 1B and fig. S1). The requirement of EZH2 for androgen-independent growth was confirmed in an in vivo mouse xenograft CRPC model using CWR22Rv1 cells (Fig. 1C).

Next, we explored EZH2-dependent genes in LNCaP and abl cells. Although similar numbers of genes were up- or down-regulated after EZH2 silencing in LNCaP cells, many more genes were significantly down-regulated upon EZH2 depletion in abl cells, and these EZH2-stimulated genes were highly expressed in abl cells (Fig. 1D). EZH2 silencing by means of two independent small interfering RNAs (siRNAs) confirmed the derepression of the EZH2-repressed gene *DAB2IP* in LNCaP cells and the down-regulation of several EZH2-stimulated genes in abl cells (fig. S2A). We found similar results in two other hormone-refractory cell lines, C4-2B and CWR22Rv1 (fig. S2B). We then examined

the profiles of EZH2-dependent genes in two clinical prostate cancer cohorts (8, 9). Although the set of EZH2-repressed genes in LNCaP cells exhibited lower expression in CRPC and marginal negative correlation with EZH2 level, the set of EZH2-stimulated genes identified in abl cells had significantly higher expression levels and positive correlation with EZH2 in these metastatic, hormone-refractory prostate tumors (Fig. 1, E and F, and fig. S3). These results suggest a potentially important functional switch of EZH2 from transcriptional repression to gene activation in CRPC.

To determine whether the gene activation function of EZH2 is the effect of direct binding, we conducted chromatin immunoprecipitation sequencing (ChIP-seq) of EZH2 and H3K27me3. Although EZH2 and H3K27me3 colocalized at the majority of sites in both LNCaP and abl cells, we identified a subset of EZH2 sites that lack nearby H3K27me3 in abl cells (Fig. 2A). These EZH2 sites lacking H3K27me3 were validated with the use of four different EZH2 antibodies (fig. S4A) and by EZH2 silencing (fig. S4B). We defined EZH2 “ensemble” peaks as those with both EZH2 and H3K27me3 enrichment, and “solo” peaks as those with only EZH2 binding. A majority of both ensemble and solo binding sites were located at the promoter regions or gene bodies (fig. S5A). Although ensemble peaks in LNCaP and abl cells overlapped considerably, very few solo peaks overlapped between the two cell lines (fig. S5B). The genes near EZH2 binding sites displayed even more striking differences (fig. S5C). This finding suggests that EZH2 gains a unique set of chromatin binding sites that lack H3K27me3 in abl cells. In addition, the solo peaks were enriched for the active histone marks H3K4me2 and H3K4me3 (i.e., dimethylation and trimethylation of Lys⁴ on histone H3) and RNA polymerase II (Pol II) (Fig. 2B), suggesting the potential function of

these peaks in gene activation. Indeed, although ensemble binding was enriched near the transcription start sites of EZH2-repressed genes, solo binding was enriched near EZH2-stimulated genes (Fig. 2C and fig. S6). Strikingly, EZH2 depletion decreased the levels of the active marks at these solo sites (fig. S7), indicating a mechanism of EZH2 in gene activation via modulation of active chromatin states. Genes directly activated by EZH2 in abl cells were significantly overexpressed in gene signatures derived from independent metastatic, hormone-refractory prostate tumors (fig. S8A), and survival analysis supported the prognostic power only of EZH2-activated genes with solo peak binding in abl

cells [Fig. 2D (8) and fig. S8, B to D (10)]. Taken together, these data support the importance of EZH2 gene activation function in CRPC.

We then tested the involvement of another PRC2 subunit, SUZ12, in the EZH2 solo peaks in CRPC. SUZ12 ChIP-seq signals displayed strong correlation with both H3K27me3 (0.57) and EZH2 ensemble peaks (0.66), but little correlation with EZH2 solo peaks (0.27) (fig. S9A). Silencing of SUZ12 drastically reduced EZH2 binding at ensemble peaks but had no effect at the solo peaks (fig. S9B). Silencing of either SUZ12 or another PRC2 core component, EED, increased the expression of the EZH2-repressed gene *DAB2IP* but had no effect on EZH2-

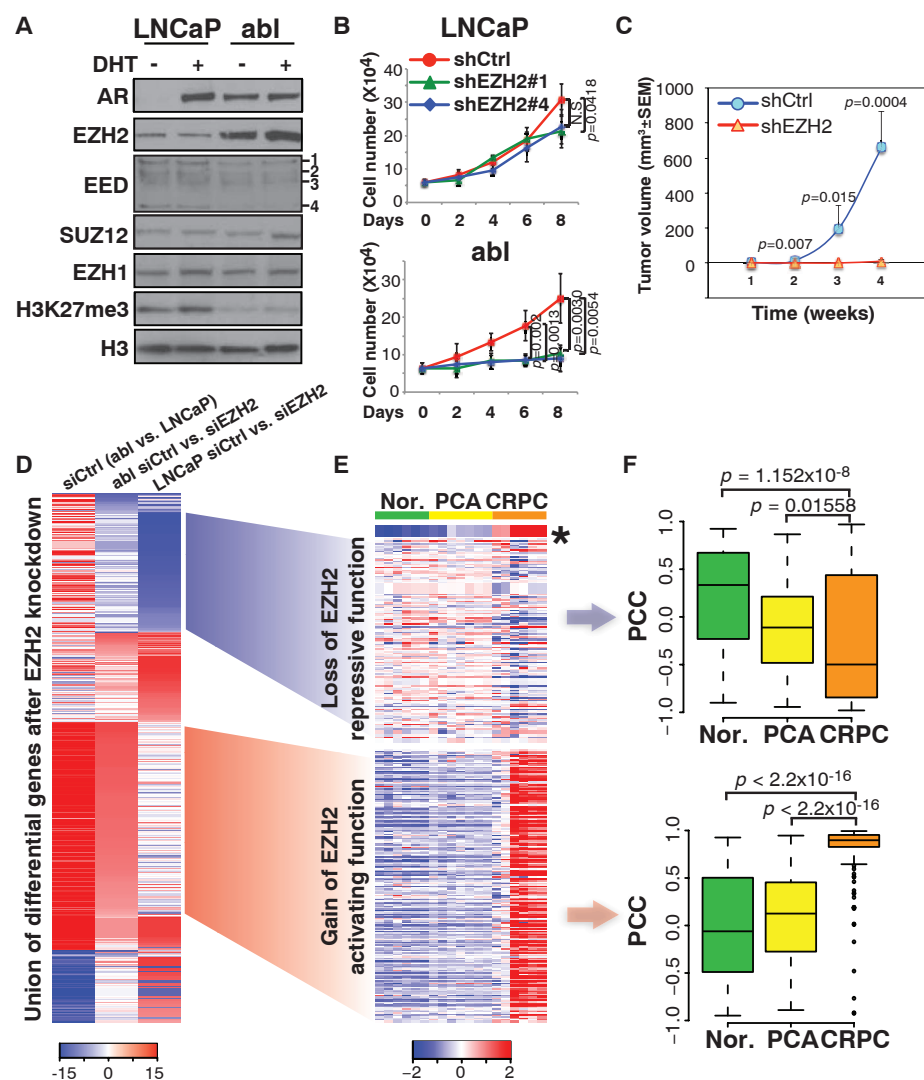


Fig. 1. Overexpression of EZH2-stimulated genes in clinical CRPC samples. **(A)** Immunoblot of nuclear extracts from LNCaP and abl cells treated without (–) or with (+) 5 α -dihydrotestosterone (DHT) to control for effects of cell growth on protein expression. EED isoforms are numbered (22). EZH1, enhancer of zeste homolog 1. **(B)** Growth of cells transduced with lentiviral short hairpin RNAs (shRNAs) targeting scrambled control (shCtrl) or EZH2 (shEZH2#1 and #4). **(C)** Tumor growth curve of castrated male *scid* mice injected with CWR22Rv1 cells with or without EZH2 silencing. **(D)** Clustering of the union of differentially expressed genes in LNCaP and abl cells after transfection with siRNAs against control (siCtrl) or EZH2 (siEZH2). **(E and F)** Heat map of expression levels (E) and box plots of Pearson correlation coefficients (PCC) (F) of EZH2-repressed (top) and EZH2-stimulated (bottom) genes with EZH2 level in the Varambally *et al.* (9) cohort. Nor., normal tissues; PCA, primary tumors. Asterisk in (E) denotes EZH2 level.

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activated genes (fig. S9C). These results indicate that EZH2 solo peaks are independent of the PRC2 complex. Results from gel filtration chromatography demonstrated that EZH2 is present in complexes other than PRC2 in abl cells, because a large fraction of EZH2 eluted as a broad peak distinct from SUZ12 or EED, although in LNCaP cells the majority of EZH2 coeluted with other PRC2 subunits (Fig. 3A).

We next asked whether the methyltransferase activity of EZH2 is required despite the lack of the other PRC2 components. We replaced the endogenous EZH2 in abl cells with either the wild type (E^{WT}) or two enzymatically inactive mutants [SET domain deletion ($E^{ST-\Delta SET}$) (11) and H694A/F672I double point mutation (E^{ST-DM}) (12, 13)] (fig. S10A). Ectopic reexpression of the wild-type EZH2, but not the catalytically inactive mutants, could rescue the effects of EZH2 silencing on both gene activation (Fig. 3B) and androgen-independent growth of CRPC cells (Fig.

3C and fig. S10B). We also found that EZH2 overexpression was sufficient to promote the androgen-independent growth of LNCaP cells and that the enzymatic activity was required (Fig. 3D). Under these conditions, the expression of EZH2-activated target genes was elevated to levels comparable to those in abl cells by wild-type EZH2, but not by the activity-dead mutants (fig. S10C). These results suggest that EZH2 makes use of a PRC2-independent methyltransferase activity for both gene activation and androgen-independent growth.

To determine how EZH2 might be targeted to solo peaks, we conducted motif analysis and found significant enrichment of the androgen receptor (AR) binding motif at EZH2 solo peaks in abl cells (fig. S11A). AR chromatin binding was enriched at the center of EZH2 solo peaks but not at ensemble peaks (fig. S11B). Coimmunoprecipitation detected a robust physical interaction between EZH2 and AR in abl cells (Fig. 3E). The interaction between AR and EZH2

was lost when the endogenous EZH2 was replaced with EZH2 deletion mutants either in Domain I (an N-terminal protein-protein interaction domain) or in the C-terminal SET domain (fig. S12), suggesting the requirement of these two domains for the interaction. EZH2 solo peaks in abl cells significantly overlapped with AR global binding, and EZH2- and AR-activated genes also overlapped significantly in abl cells (fig. S13). However, not all solo peaks contained an AR motif or overlapped with AR binding, which suggests that other factors in addition to AR may contribute to EZH2 recruitment to solo sites.

Although EZH2 depletion did not change AR mRNA or protein levels, it did decrease AR-associated lysine methylation; this required an intact EZH2 enzymatic activity (fig. S14). Such a finding suggests that EZH2 exerts its activation function not by modulating the AR level, but rather through alterations in the methylation of AR or AR-associated proteins. Silencing of EZH2 decreased AR recruitment to solo sites bound by both AR and EZH2 (fig. S15A) and had no significant effects on AR binding to other sites (fig. S15B). Similarly, knockdown of AR decreased EZH2 binding to the colocalized solo peaks (fig. S15C). Depletion of both EZH2 and AR led to a more marked reduction in the expression of co-regulated genes than silencing of either alone (fig. S15D). These results indicate that EZH2 and AR activate a set of target genes through their cooperative recruitment.

To identify what factors determine the functional switch of EZH2 from a repressor to an activator, we examined the phosphorylation status of EZH2, which has been reported to alter its enzymatic activity toward H3K27 (14–17). Phosphorylation levels at both Ser²¹ and Thr⁴⁹² were elevated in abl cells relative to LNCaP cells, whereas phosphorylation at Thr³⁵⁰ was equivalent (Fig. 4A). We replaced the endogenous EZH2 with phosphorylation site mutants to determine the potential role of site-specific phosphorylation in EZH2-mediated gene activation, and found that a Ser²¹ → Ala (S21A) mutant failed to rescue the down-regulation of EZH2-activated genes upon EZH2 silencing in abl cells (fig. S16A). In addition, only the antibody specific for phosphorylated Ser²¹ could detect EZH2 enrichment preferentially at the solo peaks (Fig. 4B and fig. S16B). Furthermore, replacement of endogenous EZH2 by wild-type EZH2 or a phosphomimetic Ser²¹ → Asp (S21D) mutant, but not the S21A mutant, could support the androgen-independent growth of CRPC cells (Fig. 4C and fig. S17, A and B). This same dependence on Ser²¹ phosphorylation was also found for the ability of EZH2 to induce the androgen-independent growth of LNCaP cells (fig. S17C). These results suggest the importance of phosphorylation at Ser²¹ in both EZH2-mediated gene activation and androgen-independent growth. We further confirmed the up-regulation of EZH2 phosphorylation at Ser²¹ in two additional hormone-refractory prostate cancer cell lines, C4-2B and CWR22Rv1 (fig. S18A). Consistent with the report

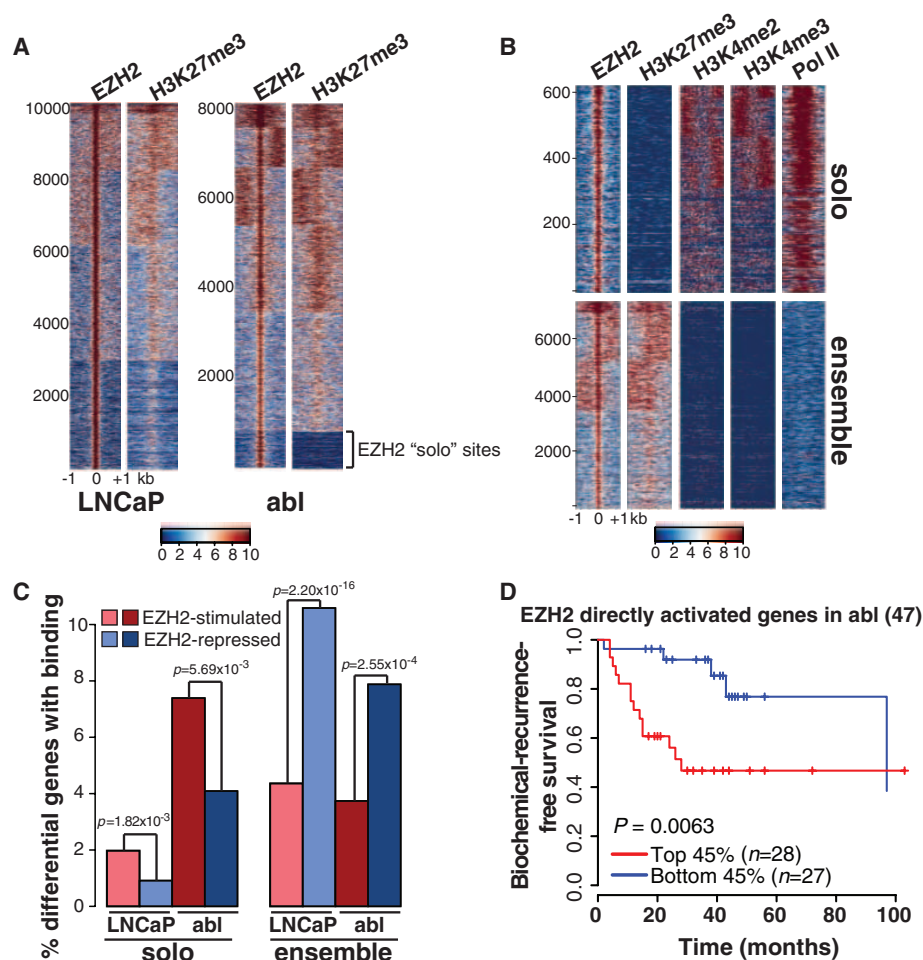


Fig. 2. EZH2 binding without H3K27me3 is associated with gene activation in CRPC. **(A)** Heat maps of EZH2 and H3K27me3 ChIP-seq signal ± 1 kb around the EZH2 peak summit in LNCaP and abl cells. The color scale indicates average signal using a 10-base pair window. The numbered index of EZH2 peaks is shown to the left. **(B)** Heat maps of EZH2, H3K27me3, H3K4me2, H3K4me3, and Pol II ChIP-seq signal ± 1 kb around EZH2 solo or ensemble peak summit in abl cells. **(C)** Percentages of differentially expressed genes upon EZH2 depletion in LNCaP or abl cells containing EZH2 solo or ensemble peaks within 20 kb around transcription start sites. **(D)** Kaplan-Meier plots of genes directly activated by EZH2 in the Yu *et al.* cohort (8). The number of genes is indicated at the top of the plot; *n*, numbers of patients.

that Akt is the kinase for EZH2 phosphorylation at Ser²¹ (16), we found higher levels of active Akt in abl cells (Fig. 4A). Although both LNCaP and abl cells were PTEN-null, the Akt phosphatase PHLPP-1 (18) was decreased in abl cells (fig. S18B), which may contribute to the activation of the phosphatidy-

linositol 3-kinase (PI3K)–Akt signaling in abl cells. To gain further insight into the involvement of EZH2 phosphorylation in the EZH2 interaction with

Fig. 3. Requirement of methyltransferase activity and interaction with AR for the EZH2 transactivation function. (A) Immunoblot of nuclear extracts from LNCaP and abl cells after gel filtration fractionation. Molecular mass standards are indicated. (B) Box plots of minimum to maximum reverse transcription polymerase chain reaction (RT-PCR) values for EZH2-activated genes in abl cells after replacement with wild-type or mutant EZH2 as indicated. (C and D) Growth of abl cells (C) and LNCaP cells (D) in hormone-depleted medium after replacement with wild-type or mutant EZH2 as indicated. (E) Coimmunoprecipitation of EZH2 and AR in LNCaP and abl cells without (–) or with (+) DHT.

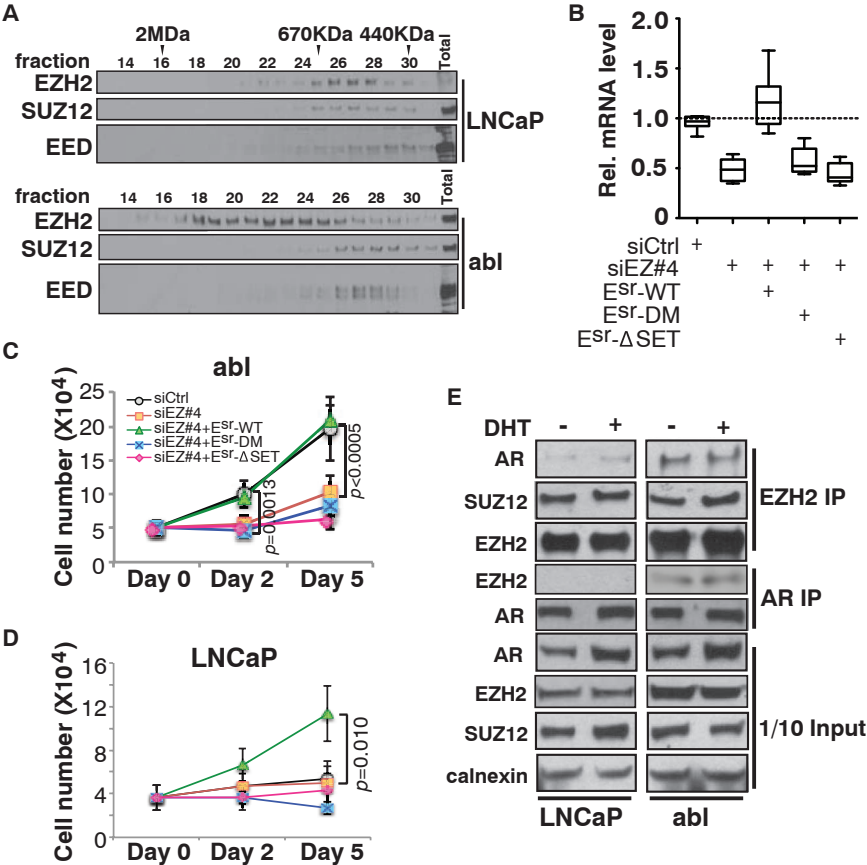
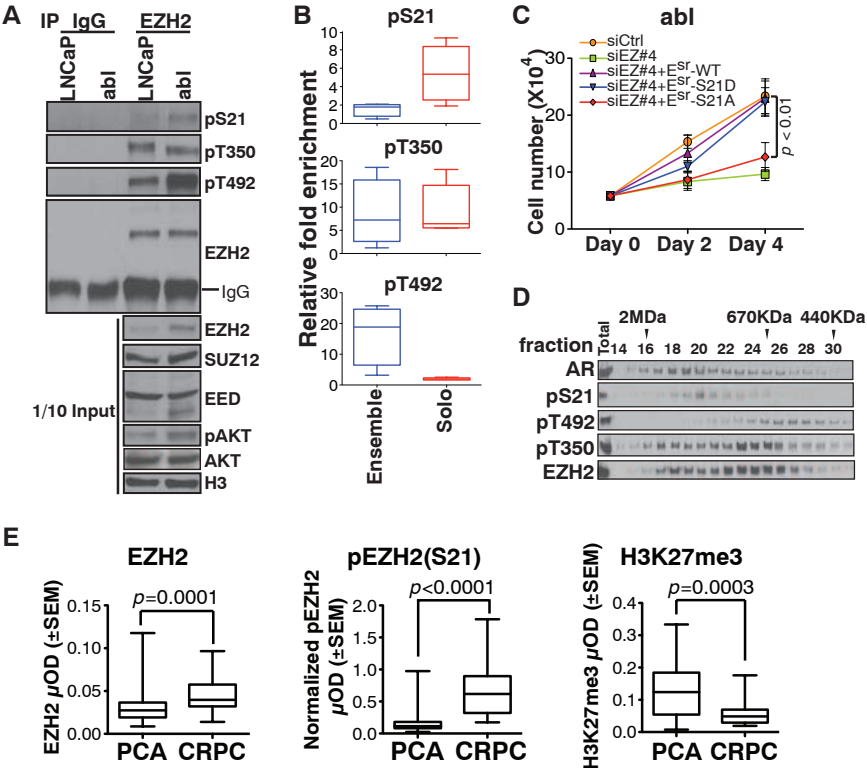


Fig. 4. Critical role of EZH2 phosphorylation at Ser²¹ for its functional switch. (A) Immunoprecipitation (IP) in LNCaP and abl cells, using control immunoglobulin (IgG) or antibodies specific to EZH2 followed by immunoblotting with the indicated antibodies. (B) Box plots of ChIP–quantitative PCR values for EZH2 recruitment to selected ensemble or solo sites, using phosphorylation-specific antibodies. (C) Growth of abl cells in androgen-depleted medium after replacement with wild-type or mutant EZH2 as indicated. (D) Immunoblot of nuclear extracts from abl cells after gel filtration fractionation. Molecular mass standards are indicated. (E) Analysis of EZH2, Ser²¹-phosphorylated EZH2 [pEZH2(S21)], and H3K27me3 protein levels by quantitative immunohistochemistry in neoadjuvant prostate tumors (PCA) and CRPC.



AR, we investigated the copurification of phosphorylated forms of EZH2 and AR by gel filtration and found that pS21 EZH2 predominantly coeluted with AR in a high-molecular weight complex (Fig. 4D). These results suggest a potential role for EZH2 phosphorylation at Ser²¹ to promote its association with an AR-containing complex.

The importance of EZH2 phosphorylation at Ser²¹ in prostate cancer progression was further analyzed by immunohistochemistry in tissue microarrays containing early-stage prostate tumors from a neoadjuvant androgen deprivation therapy trial and metastatic, hormone-refractory tumors (Fig. 4E and fig. S19). As previously reported (1), the level of EZH2 in CRPC was higher than during early-stage disease, and pS21 EZH2 was even more significantly increased in CRPC. Intriguingly, H3K27me3 levels significantly decreased with prostate cancer progression, consistent with our observation that the global level of H3K27me3 in abl cells was considerably lower than in LNCaP cells (Fig. 1A). This result further supports our conclusion that the oncogenic activity of EZH2 in CRPC is independent of its Polycomb-repressive function.

This study demonstrates that phosphorylation of EZH2 at Ser²¹, mediated directly or indirectly by the PI3K-Akt pathway, can switch its function from a Polycomb repressor to a transcriptional coactivator of AR (and potentially other factors). Rescue experiments and the lack of correlation with H3K27me3 levels support a role for EZH2-directed methylation of sub-

strates other than H3K27, including potential nonhistone proteins. The current rationale for EZH2 inhibitor design is based primarily on targeting its Polycomb-repressive activity and uses H3K27me3 as the pharmacodynamic readout (19). However, the observed loss-of-function mutations of EZH2 in myelodysplastic syndrome and acute leukemia raise concerns that such inhibitors might exhibit important hematologic side effects (20, 21). Our finding of an altered function for EZH2 in CRPC cells raises the potential to develop inhibitors that specifically target the EZH2 activation function while sparing its PRC2-repressive function. In addition, our finding that EZH2 cooperates with AR-associated complexes and requires phosphorylation to support CRPC growth suggests novel combination therapies for the treatment of metastatic, hormone-refractory prostate cancer (fig. S20).

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Supplementary Materials

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Materials and Methods
Figs. S1 to S20
Tables S1 to S4
References (23–44)

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Airn Transcriptional Overlap, But Not Its lncRNA Products, Induces Imprinted *Igf2r* Silencing

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Mammalian imprinted genes often cluster with long noncoding (lnc) RNAs. Three lncRNAs that induce parental-specific silencing show hallmarks indicating that their transcription is more important than their product. To test whether *Airn* transcription or product silences the *Igf2r* gene, we shortened the endogenous lncRNA to different lengths. The results excluded a role for spliced and unspliced *Airn* lncRNA products and for *Airn* nuclear size and location in silencing *Igf2r*. Instead, silencing only required *Airn* transcriptional overlap of the *Igf2r* promoter, which interferes with RNA polymerase II recruitment in the absence of repressive chromatin. Such a repressor function for lncRNA transcriptional overlap reveals a gene silencing mechanism that may be widespread in the mammalian genome, given the abundance of lncRNA transcripts.

Macro long noncoding (lnc) RNAs such as *Airn* (1), *Kcnq1ot1* (2), or *Nespas* (3) that silence imprinted gene clusters offer important epigenetic models for the numerous lncRNAs mapped in the mammalian genome (4–6). In the *Igf2r* imprinted cluster, the paternally expressed *Airn* (antisense *Igf2r* RNA non-coding) macro lncRNA silences in cis the paternal

alleles of *Igf2r*, *Slc22a3*, and *Slc22a2* (1). *Airn* may use different silencing mechanisms, because *Igf2r* is silenced in all embryonic, extraembryonic, and adult tissues that express *Airn*, whereas *Slc22a2* and *Slc22a3* are only silenced in some extraembryonic lineages (7, 8). In support of this, *Slc22a3* silencing in the placenta depends on the *Airn* lncRNA product recruiting EHMT2 histone methyl-

transferase, whereas *Igf2r* silencing does not (9). *Igf2r* silencing is also not dependent on Polycomb-group proteins or DNA methylation (10, 11). Thus, the mechanism by which *Airn* silences *Igf2r*, the only gene in this cluster with an essential embryonic function (12), remains unknown. *Airn* transcription overlaps the *Igf2r* promoter but not the *Slc22a3* or *Slc22a2* promoters (fig. S1A), indicating that silencing could depend on *Airn* transcriptional overlap independent of the *Airn* lncRNA product.

To test the role of *Airn* transcription versus product in *Igf2r* silencing, we used homologous recombination in embryonic stem (ES) cells to insert polyadenylation (polyA) cassettes on the paternal chromosome that truncate *Airn* to different

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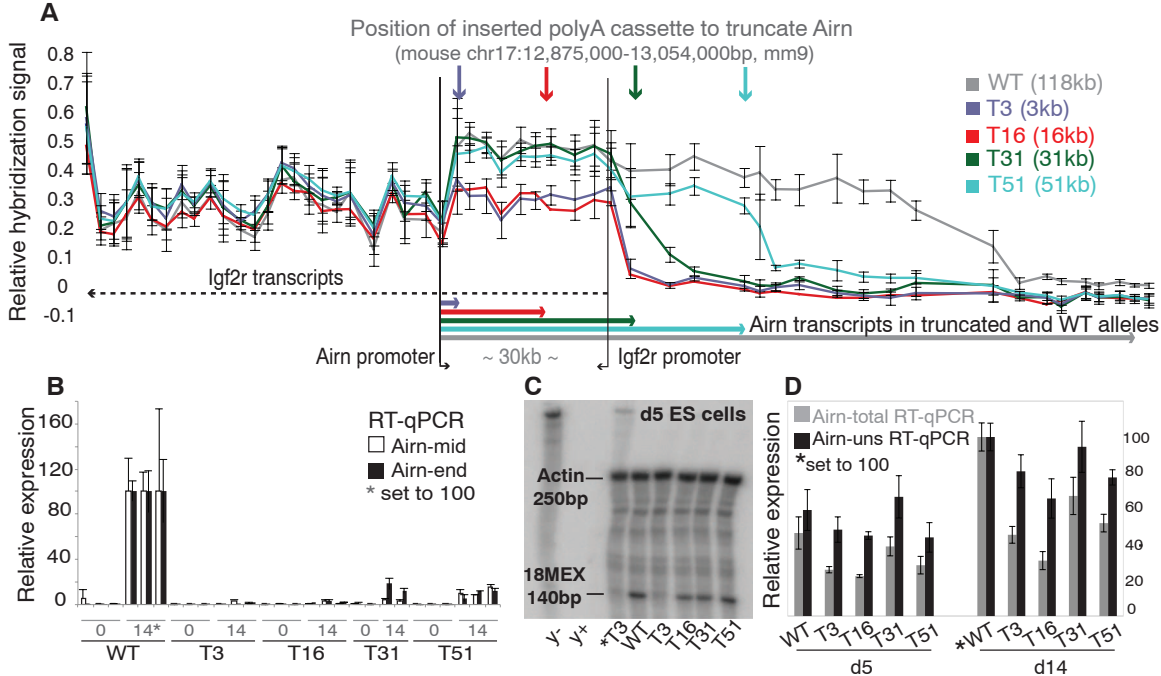
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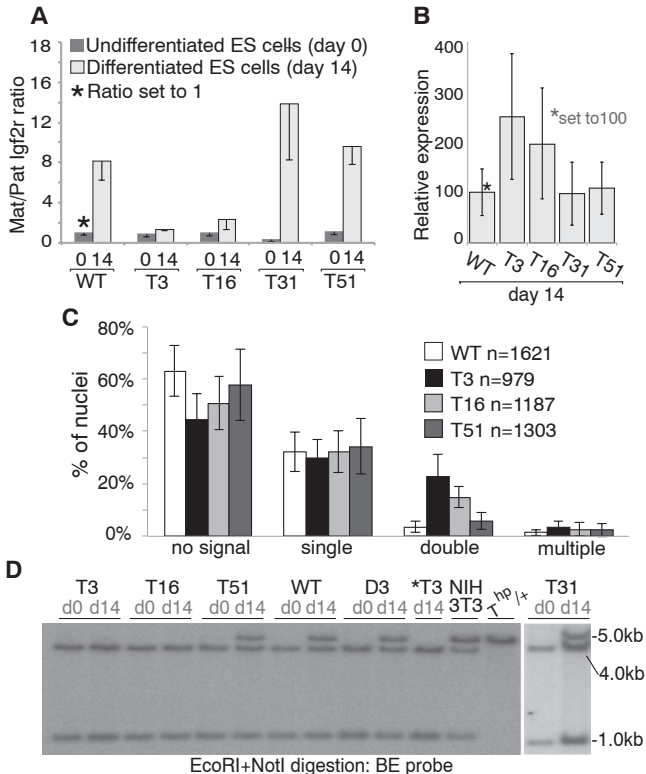
Fig. 1. *Airn* is shortened to different lengths by a targeted polyA cassette. (A) Tiling arrays confirm *Airn* loss upstream of *Igf2r* in truncated alleles and absence of novel spliced products. Data are relative hybridization intensity plots (13); error bars are means $\pm$ SD of 21 windows of the averaged signal from nine tiled oligos. (B) Loss of *Airn* 53.2 kb (*Airn*-mid) and 98.7 kb (*Airn*-end) upon polyA insertion 3, 16, 31, and 51 kb downstream from the *Airn* TSS in day 14 differentiated (d14) cells. *Airn* is not expressed in undifferentiated (d0) ES cells. (C) RNase protection shows normal *Airn* levels 7.4 kb from the TSS in wild type (WT), T16, T31, and T51 and its loss in T3. Actin was used as a loading control. y⁻, probe lacking RNase; y⁺, probe plus RNase; *T3, MEFs with a previously generated 3-kb truncation (1). (D) Reduced spliced + unspliced *Airn* (*Airn*-



lengths (figs. S1 to S5), (13). ES cell differentiation was used to recapitulate the developmental onset of *Airn* and *Igf2r* imprinted expression (14) (fig. S1B). PolyA cassettes inserted before (T3, T16) or after (T31, T51) the *Igf2r* promoter truncated the 118-kb *Airn* to 3, 16, 31, and 51 kb, respectively (Fig. 1). RNA tiling array hybridization (Fig. 1A) demonstrated *Airn* truncation and the absence of novel spliced variants in all alleles. Although *Airn* was lost downstream, normal levels of unspliced *Airn* were maintained upstream of each truncation site (Fig. 1, B to D, and fig. S3B). Wild-type *Airn* is mostly unspliced, but 5% of nascent transcripts are spliced to four variants (fig. S1A) that constitute ~30% of steady-state *Airn* because of their high stability (15). All four truncation alleles showed ~40% loss of total *Airn*; this reflects a loss of spliced products, as splice acceptors lie downstream of each truncation (Fig. 1D). Together, the truncations of *Airn* at 3, 16, 31, and 51 kb removed 97.5, 86.5, 73.8, and 56.8% of the 118-kb *Airn* product, respectively, including all spliced variants. Furthermore, the truncations did not change *Airn* expression kinetics or its characteristic inefficient splicing, nor did they interfere with the methylation-free state of its paternal promoter (Fig. 1D and fig. S3C).

We next tested whether *Airn* truncation alleles silence the paternal *Igf2r* promoter. *Igf2r* allelic expression was analyzed using two polymerase chain reaction (PCR) assays for steady-state expression. Undifferentiated ES cells showed the expected biallelic *Igf2r* expression in the absence of *Airn* expression (14) (Fig. 2A and fig. S6A). Upon differentiation, T3 and T16 cells

Fig. 2. The greater part of the *Airn* lncRNA product is not required for repression of *Igf2r*. (A) Allele-specific reverse transcription quantitative PCR (RT-qPCR) showing *Igf2r* imprinted expression in WT, T31, and T51, but not in T3 or T16, indicated by increased maternal/paternal *Igf2r* ratio in differentiated/d14 ES cells compared to a ratio of ~1 in undifferentiated (d0) cells. Bars show means $\pm$ SD of one to three biological replicates, each with three technical replicates (13). (B) RT-qPCR showing total steady-state *Igf2r* in T3, T16, T31, and T51 relative to WT cells; means $\pm$ SD as in (A). (C) *Igf2r* RNA FISH in differentiated (d5) cells shows loss of imprinted *Igf2r* expression, indicated by increased numbers of cells with double signals in T3 and T16 compared to T51 or WT (fig. S6C shows representative images). Discontinuous transcription of active genes (20) results in many nuclei with no signal using intronic probes. Single spots, imprinted or stochastic biallelic expression; double spots, biallelic expression; *n*, nuclei counted; bars, total counts of two biological replicates and two technical replicates (13). (D) The paternal *Igf2r* promoter is methylated in differentiated WT, T31, and T51 but not in T3 or T16 cells or in undifferentiated (d0) ES cells, as shown by the 5-kb *EcoRI* fragment resistant to *NotI* digestion. *T3, E14 ES cells with a 3-kb *Airn* truncation (2); NIH3T3, MEFs with both parental alleles; *T^{hi}*+, uniparental MEFs. T31 cells were assayed on a separate gel.



maintained biallelic *Igf2r* expression, whereas T31 and T51 cells showed a gain of *Igf2r* imprinted expression similar to wild-type cells. This resulted in a factor of ~2 *Igf2r* increase in T3 and T16 cells relative to wild-type, T31, and T51 cells

(Fig. 2B). The T3 truncation has been examined in a mouse model (1), which validates the ES cell model used here. The data also show that the T3 and T16 truncations do not interfere with *Igf2r* expression, as the derepressed paternal and

wild-type maternal alleles expressed similar *Igf2r* levels. Similarly, the T31 truncation cassette inserted on the maternal chromosome allows wild-type *Igf2r* expression (fig. S3A). Lastly, RNA fluorescence in situ hybridization (FISH) demonstrated loss of *Igf2r* imprinted expression at a transcriptional level in T3 and T16 but not in T51 cells (Fig. 2C and fig. S6C). Repression of the paternal *Igf2r* allele is accompanied by gain of DNA methylation on its promoter CpG island (CGI) (14). This methylation is absent in T3 and T16 but is present in T31 and T51 differentiated cells (Fig. 2D and fig. S6B). The T31 allele represses *Igf2r* and truncates before the first *Airn* splice acceptor at 37 kb, showing that all spliced *Airn* variants are unnecessary for *Igf2r* silencing. The T3 and T16 truncations show that the first 16 kb of *Airn* are insufficient to silence *Igf2r*, and the T31 and T51 truncations show that the last 87 kb of *Airn* are unnecessary. Together, they localize *Airn* repressor activity to the remaining 12.7% between the T16 and T31 truncations (fig. S6D). Because this region contains the *Igf2r* promoter, the data support the hypothesis that repressor activity results from *Airn* transcription.

The nuclear size of the *Airn* lncRNA product correlates with silencing *Slc22a3* in the placenta (9). We used RNA FISH to test whether *Airn* nuclear size or its subnuclear localization correlates with *Igf2r* silencing in embryonic cells. Both parameters showed no difference between

Fig. 3. *Airn* macro lncRNA size and location do not determine *Igf2r* repression. (A) The size of the *Airn* RNA FISH signal (white arrow) relative to the nucleus [ring identified by 4',6-diamidino-2-phenylindole (DAPI)] is similar for T51 and T16 but different from WT. Horizontal line denotes median; *P* values are results of *t* tests using two biological replicates performed in two technical replicates (13); *n*, number of nuclei. (B) Similar subnuclear localization for WT, T51, and T16 alleles. The nuclear area was binned into pseudo-colored inner, middle, and outer circles with equal spacing, and percentages of *Airn* RNA FISH signals (red arrow) in different distance bins were scored using the same data set as in (A).

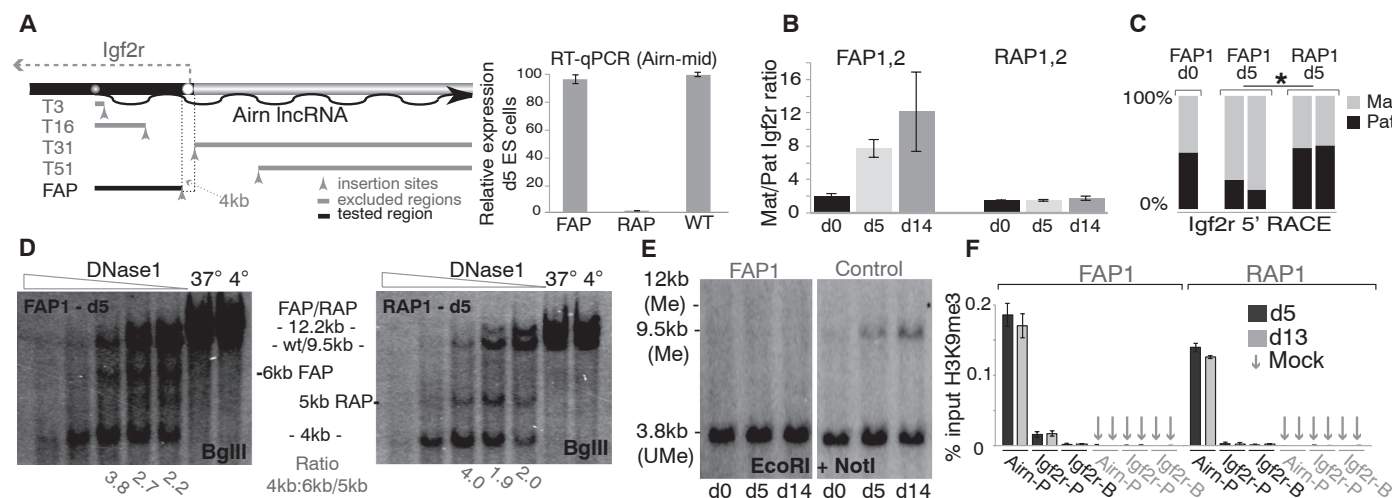
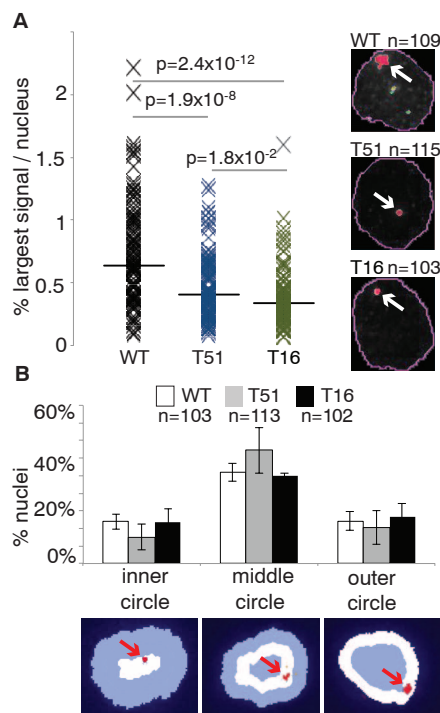


Fig. 4. *Airn* transcriptional overlap is sufficient for *Igf2r* repression. (A) Left: Map showing parts of the *Airn* product excluded by T3, T16, T31, and T51 truncation alleles and the region tested by the FAP allele. Right: Wild-type levels of *Airn* are expressed from the FAP but not the RAP allele (means $\pm$ SD of three technical replicates). (B) FAP but not RAP cells maintain imprinted *Igf2r* expression. cDNA sequence single-nucleotide polymorphism (SNP) quantitation (fig. S10C) shows increased maternal/paternal ratio in FAP but not RAP cells (means $\pm$ SD of four biological replicates). (C) The repressed FAP *Igf2r* promoter has reduced levels of capped mRNA. cDNA sequence SNP quantitation shows the ratio of maternal/paternal capped *Igf2r* mRNA in day 5 differentiated (d5) FAP and RAP cells (mean of two sequence reads per bar; **P* = 0.0001, *t* test). (D) Chromatin accessibility in DNase I-treated FAP1 and RAP1 nuclei; blot was hybridized with probe-NE4 to identify the

Igf2r promoter (image levels are nonlinearly adjusted to improve visualization). The repressed FAP *Igf2r* promoter (6-kb FAP-DNase I/BglII fragment) has open chromatin similar to the active RAP promoter (5-kb RAP-DNase I/BglII fragment) (fig. S12A). (E) DNA methylation in genomic DNA digested with BglII and methyl-sensitive NotI, hybridized with probe-NE4. Absence of a 12-kb BglII fragment in FAP cells indicates an unmethylated (Ume) silent paternal *Igf2r* promoter (fig. S6B). In control R2Δ/+ cells, the 9.5-kb fragment indicates normal methylation (Me) of the silent paternal *Igf2r* promoter in differentiated ES cells. (F) H3K9me3 chromatin immunoprecipitation qPCR on *Airn* and *Igf2r* promoters (P) and *Igf2r* intron1 (B) in FAP (silenced paternal *Igf2r* promoter) and RAP (active paternal *Igf2r* promoter) cells that also contain a silenced maternal *Airn* promoter. Data are means $\pm$ SD of three technical replicates.

T51, which silences *Igf2r*, and T16, which does not (Fig. 3A and fig. S7). The larger size of wild-type *Airn* is therefore unrelated to *Igf2r* silencing. The majority of FISH signals lay in the mid-nuclear plane, with both repressing and non-repressing *Airn* alleles showing a similar localization (Fig. 3B) and a similar relative position to the nucleolus (fig. S7). Together, these data indicate no organizational role for the *Airn* product in *Igf2r* silencing, thereby supporting claims (9) that *Airn* silences *Igf2r* and *Slc22a3* by different mechanisms.

A prediction of a transcriptional overlap model is that the interfering promoter should impose repressor activity. To test this, we moved the *Airn* promoter ~700 base pairs before the *Igf2r* transcription start site (TSS) in ES cells that lack an endogenous paternal *Airn* promoter (16) (figs. S8 and S9). FAP (forward *Airn* promoter) cells contain the repositioned *Airn* promoter and the first 1.8 kb of the *Airn* lncRNA product (also present in the T3 and T16 alleles that do not silence *Igf2r*) in wild-type orientation, and express normal levels of *Airn* that overlap the paternal *Igf2r* promoter (Fig. 4A and fig. S9B). RAP (reverse *Airn* promoter) cells contain an inverted repositioned *Airn* promoter and do not transcribe *Airn* over the paternal *Igf2r* promoter. Undifferentiated ES cells showed biallelic *Igf2r* expression in FAP or RAP cells (Fig. 4B and fig. S10A) similar to that seen in wild-type cells (Fig. 2A). Upon differentiation, RAP cells maintained biallelic *Igf2r* expression but FAP cells showed paternal-specific *Igf2r* silencing with a maternal/paternal *Igf2r* ratio similar to that in wild-type and T31 and T51 truncated cells. The FAP allele shows that the *Airn* promoter imposes repressor activity and also excludes the 11-kb *Airn* region spanning the T16 to FAP insertion sites. Together with the truncation alleles, this excludes 96.7% of the *Airn* lncRNA product as necessary for silencing *Igf2r*. The FAP and T31 alleles that both silence *Igf2r* have in common a 4-kb *Airn* product that overlaps the *Igf2r* promoter (Fig. 4A). Another prediction of the transcriptional overlap model is that repressor activity is maintained if the *Igf2r* promoter is substituted. We previously replaced this 4-kb region in vivo with a *Tk-neo* reporter gene that preserves imprinted expression and methylation (17). To exclude the possibility that the *Tk-neo* reporter fortuitously reconstituted endogenous elements, we demonstrated that it lacks any nucleotide or structural similarity to this region (fig. S11). Thus, this 4-kb endogenous *Airn* product is unnecessary to silence the *Igf2r* promoter.

These data are consistent with *Airn* silencing the *Igf2r* promoter by transcriptional interference, which reduces recruitment of functional RNA polymerase II (RNAPII) to the *Igf2r* promoter, independent of *Airn* lncRNA products. In FAP cells, the proximity of the repositioned *Airn* promoter prevents a direct analysis of RNAPII on the repressed *Igf2r* promoter. To circumvent this, we assayed for S5P-RNAPII-dependent capped

Igf2r mRNA and found that it was reduced in FAP but not in RAP cells (Fig. 4C and fig. S10B). Transcriptional interference models (18) predict suppression of the “sensitive” promoter by an “interfering” promoter, initially in the absence of repressive chromatin. The repressed FAP *Igf2r* promoter maintained features associated with active chromatin, such as a strong DNase I–hypersensitive site (Fig. 4D and fig. S12, A and B) and H3K4me3 (fig. S12C), similar to the active RAP *Igf2r* allele. The wild-type paternal *Igf2r* promoter is modified late in development by DNA methylation that is unnecessary for *Igf2r* repression in embryo or placenta (10) and by H3K9me3 (15, 19), which is unnecessary for *Igf2r* silencing in the placenta (9). The repressed FAP *Igf2r* promoter remained free of DNA methylation (Fig. 4E), possibly due to the proximity of the repositioned *Airn* promoter CGI. Low-level H3K9me3 was less than on the silent *Airn* promoter by a factor of 10, similar to ratios in mouse embryonic fibroblasts (MEFs) (19), (Fig. 4F). Together, these data show that *Airn* transcriptional overlap interferes with functional RNAPII recruitment to the *Igf2r* promoter in the presence of active chromatin, supporting a model whereby *Airn* induces silencing by transcriptional interference (fig. S12D).

Collectively, our data demonstrate a role for *Airn* transcription, but not its spliced or unspliced lncRNA products, in silencing the *Igf2r* promoter. The demonstration that *Igf2r* silencing depends on *Airn* transcription reflects hallmark features of macro lncRNAs, such as inefficient splicing, extreme length, high repeat content, lack of conservation, and short half-life (15), which all indicate that transcription is more important than product. It is not yet known how many of the growing number of mammalian lncRNAs share these hallmarks. If they do, the

range of lncRNA functions in the mammalian genome could be substantially enlarged.

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Supplementary Materials

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Materials and Methods

Figs. S1 to S12

References (21, 22)

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A Steroid Receptor–MicroRNA Switch Regulates Life Span in Response to Signals from the Gonad

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Although the gonad primarily functions in procreation, it also affects animal life span. Here, we show that removal of the *Caenorhabditis elegans* germ line triggers a switch in the regulatory state of the organism to promote longevity, co-opting components involved in larval developmental timing circuits. These components include the DAF-12 steroid receptor, which is involved in the larval stage two–to–stage three (L2–L3) transition and up-regulates members of the *let-7* microRNA (miRNA) family. The miRNAs target an early larval nuclear factor *lin-14* and *akt-1*/kinase, thereby stimulating DAF-16/FOXO signaling to extend life. Our studies suggest that metazoan life span is coupled to the gonad through elements of a developmental timer.

Studies of the nematode *Caenorhabditis elegans* have shown that these animals live up to 60% longer when germline stem

cells (GSCs) are eliminated from the gonad (1, 2). This longevity depends on the presence of the somatic gonad, suggesting a model wherein

GSCs antagonize life-lengthening signals from the somatic gonad. Similar to *C. elegans*, work with *Drosophila* demonstrates that animals lacking GSCs live longer than controls (3), suggesting that gonadal longevity is evolutionarily conserved. Although the life-shortening signal(s) from the germ line remain elusive, life-lengthening signals from the somatic gonad include bile acid-like steroids, called dafachronic acids (DAs), which activate the steroid hormone receptor DAF-12, a homolog of vertebrate liver-X, farnesoid-X, and vitamin-D receptors (4, 5). How the DAs themselves are regulated and activate downstream targets remains unclear. Evidence indicates that DA/DAF-12 signaling regulates genes important for longevity and also converges on the DAF-16/FOXO transcription factor by potentiating nuclear localization and augmenting transcriptional activity on longevity-promoting genes (6, 7). However, the mechanisms coupling these pathways are unknown. DAF-16/FOXO is also stimulated

independently by decreased insulin/insulin-like growth factor (IGF) receptor (IR) signaling, because the longevity of *daf-2*/IR and germline-less *glp-1* mutants is additive (1).

To elucidate how germline loss increases longevity, we first asked whether it affects regulation of DA signaling. When we examined mRNA levels of DA signaling components by quantitative polymerase chain reaction (PCR), we did not observe any differences between germline-less *glp-1* mutants and gonad-intact wild-type (WT) animals at the third larval stage (L3). However, by L4 and day 1 of adulthood (D1), the hormone biosynthetic gene *daf-36*/Rieske-oxygenase was significantly up-regulated in *glp-1* (Fig. 1A and fig. S1D, as well as supplementary materials and methods) but down-regulated in the wild type (Fig. 1A). Other DA-biosynthetic genes, including *daf-9*/CYP27A1 and *dhs-16*/HSD, were less affected (fig. S1A) (8). Expressed mainly in the intestine, *daf-36* catalyzes the first step in Δ^7 -DA biosynthesis, converting cholesterol to 7-dehydrocholesterol (9, 10). Accordingly, 7-dehydrocholesterol and Δ^7 -DA were increased four- to fivefold in *glp-1* animals, as measured by gas chromatography–tandem mass spectrometry (Fig. 1, B and C). In D1 adults, *daf-36* up-regulation was largely independent of *daf-12* and *daf-16* (fig. S1, B and C). These data suggest that a regulatory switch governs DA signaling in response to signals from the reproductive

system and reveal that germline loss stimulates the DA signaling pathway.

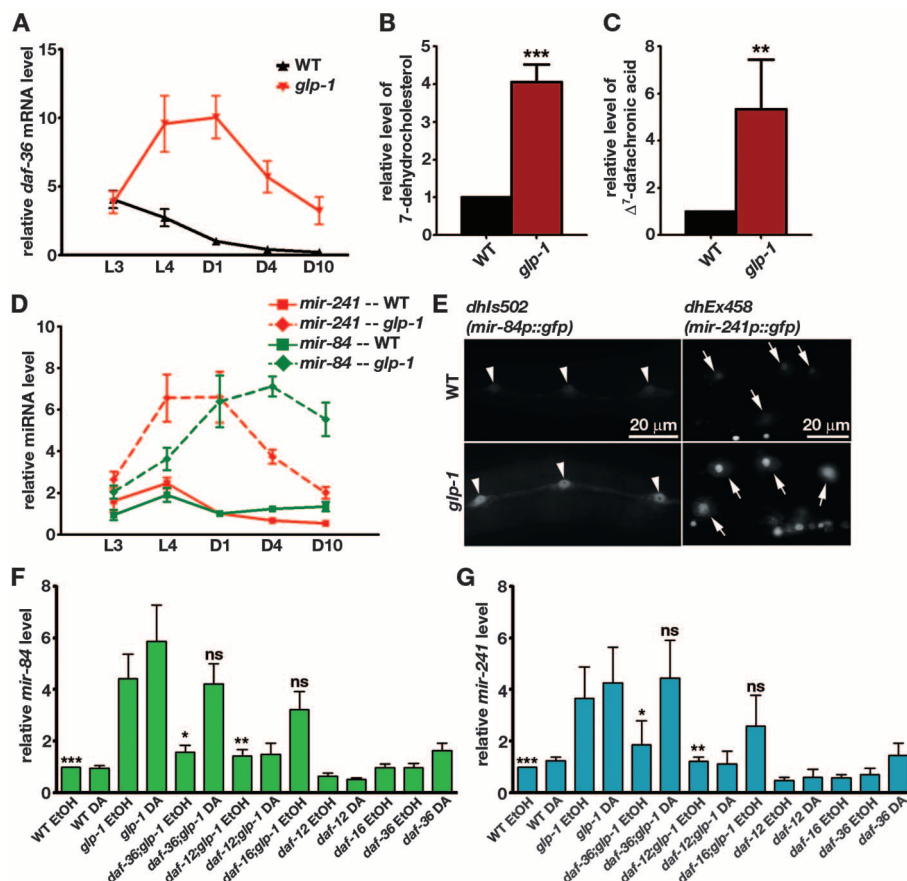
To see whether germline loss stimulates DAF-12 transcriptional activity, we focused on *let-7*-related microRNAs (miRNAs), *mir-84* and *mir-241*, which are direct DAF-12 targets in larval developmental timing pathways during L2-L3 transitions (11, 12). Indeed, miRNA expression increased in *glp-1* mutants by the L4 stage and peaked at three- to fourfold by D1 (Fig. 1, D and E). miRNA up-regulation was DA- and *daf-12*-dependent, whereas *daf-16* or *nhr-80*, an HNF4-like nuclear receptor regulating gonadal longevity (13), had little effect (Fig. 1, F and G, and fig. S2F). Consistently, *mir-84p::gfp* and *mir-241p::gfp* promoter constructs also exhibited transcriptional up-regulation in *glp-1* mutants, particularly in epidermal seam cells (*mir-84*) and intestinal cells (*mir-241*), revealing complex tissue-specific regulation (Fig. 1E and fig. S2E). Other members of this miRNA family, *let-7* and *mir-48*, were also up-regulated but showed no clear DA/DAF-12 dependence. By contrast, an unrelated miRNA, *mir-228*, decreased during the same time frame (fig. S2). We conclude that germline absence up-regulates DA signaling, accompanied by transcriptional activation of *let-7* family members, including the DAF-12 target genes *mir-84* and *mir-241*.

To test whether the miRNAs function in the gonadal longevity pathway, we removed GSCs

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Fig. 1. Ablation of the germ line up-regulates DA/DAF-12 signaling. **(A)** Time course of *daf-36* mRNA level in WT and germline-less animals (*glp-1*). Error bars denote SEM. **(B and C)** 7-dehydrocholesterol (B) and Δ^7 -DA (C) are elevated in germline-less animals. *t* test; ***P* < 0.01, ****P* < 0.001. **(D)** Time course of *mir-84* and *mir-241* levels in WT and *glp-1* animals. **(E)** In *glp-1* animals, *mir-84p::gfp* and *mir-241p::gfp* expression are up-regulated in seam cells (arrowheads) and intestine (arrows), respectively. **(F and G)** Germline absence results in up-regulation of *mir-84* and *mir-241* in a DAF-12/DA-dependent manner. Strains were grown on plates supplemented with ethanol (EtOH) or Δ^4 -dafachronic acid (DA). Analysis of variance (ANOVA) versus *glp-1* EtOH; ns, nonsignificant. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.



by laser microsurgery in WT and *mir-84;mir-241* mutants. As expected, life span was extended in germline-ablated WT animals compared with mock-ablated controls. Whereas gonad-intact *mir-84;mir-241* controls resembled WT animals, life-span extension was abolished in germline-ablated *mir-84;mir-241* double mutants (Fig. 2A). Similarly, miRNA loss suppressed longevity and stress resistance in *glp-1* mutants (fig. S3 and table S1). By contrast, *mir-84;mir-241* mutation had little effect on longevity caused by reduced mitochondrial function (*cco-1RNAi*; RNAi, RNA interference) or IR signaling (*daf-2RNAi*) (Fig.

2B). *mir-84* and *mir-241* transgenes driven by endogenous promoters restored stress resistance and longevity in *mir-84;mir-241;glp-1* triple mutants but did not significantly extend life span in gonad-intact animals (fig. S3, B and C, and table S1). Thus, *mir-84* and *mir-241* are specifically required but not sufficient for life extension in the gonadal pathway. *mir-48* mutants also significantly decreased *glp-1* longevity, but affected WT animals as well (table S1). *let-7* mutants were not analyzed because of their severe developmental defects. Therefore, we focused on *mir-84* and *mir-241* for further analysis.

DAF-16/FOXO is essential for longevity in the gonadal pathway. In germline-less animals, this transcription factor accumulates in intestinal nuclei, where it regulates genes important for life-span extension (1, 6). Both DAF-12 and DAF-36 promote DAF-16 nuclear localization (4, 6); DAF-12 and DAF-16 share transcriptional responsibilities for longevity (6). To investigate whether the miRNAs interact with DAF-16, we analyzed life span upon *daf-16RNAi*. If miRNA deficiency reduces *glp-1* longevity by a mechanism independent of *daf-16*, then *mir-84;mir-241;glp-1;daf-16RNAi* animals should live even shorter

Fig. 2. DAF-12 target miRNAs are required for gonadal longevity through DAF-16/FOXO. (A) *mir-84* and *mir-241* are required for gonadal longevity. Laser microsurgery was performed to ablate the germ line. (B) *mir-84* and *mir-241* do not affect the longevity by reduced IR signaling (*daf-2RNAi*) or mitochondrial function (*cco-1RNAi*). (C) Life-span analysis of indicated strains on *daf-16RNAi*. (D) Quantitative real time PCR of DAF-16/FOXO target genes (*T08B1.1* and *btb-14*) in worms of indicated genotypes. ANOVA; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

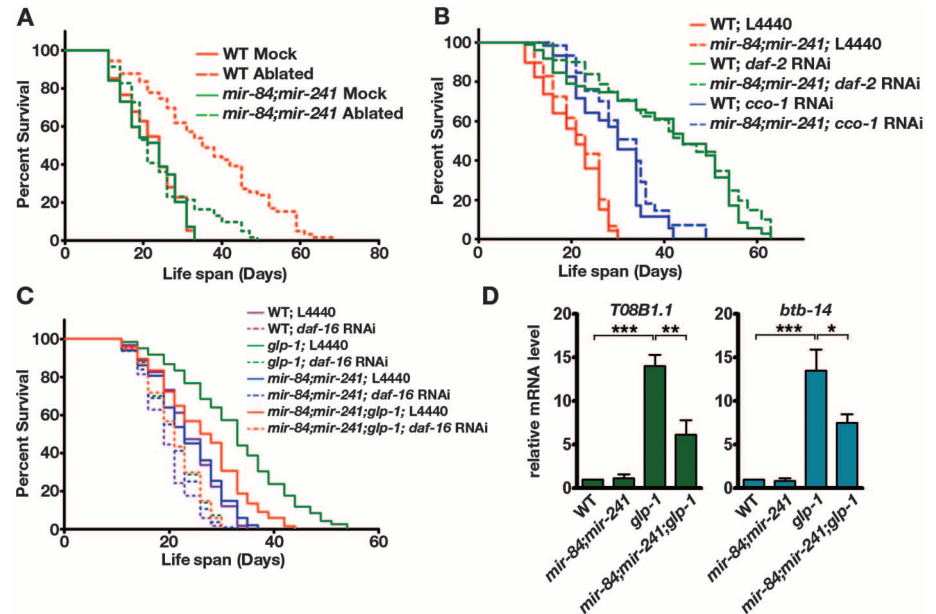
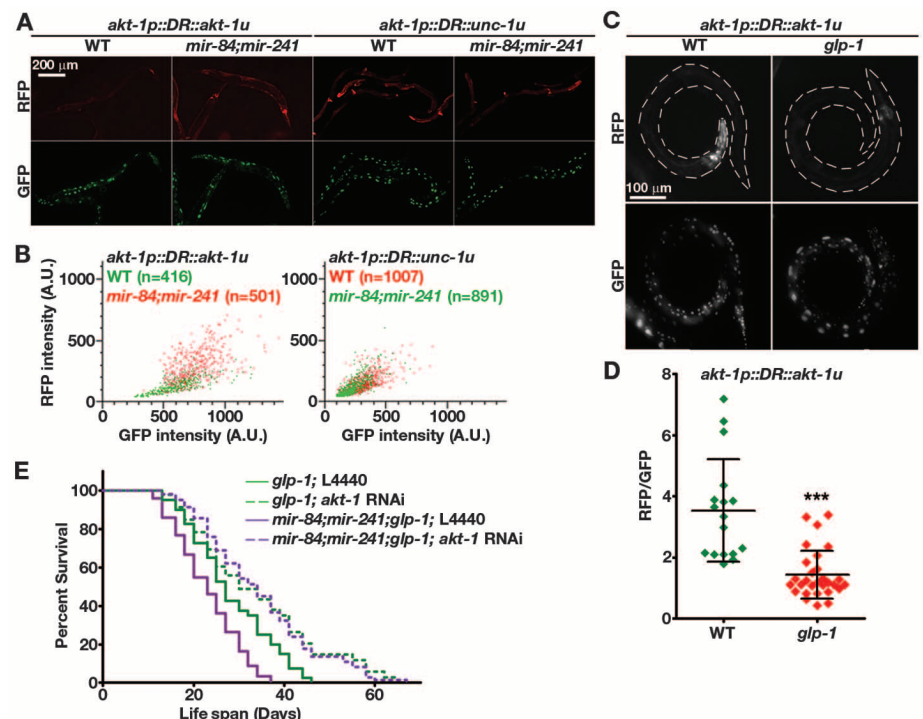


Fig. 3. *akt-1* kinase is a miRNA target influencing gonadal longevity. (A and B) *mir-84* and *mir-241* target the 3'UTR of *akt-1* but not *unc-1* in vivo. Although GFP driven by the *akt-1* promoter in the DR construct remains similar, red fluorescent protein controlled by the *akt-1* 3'UTR is significantly elevated in *mir-84;mir-241*. (C and D) *akt-1*-3'UTR-DR is down-regulated in germline-less animals. 17 WT and 29 *glp-1* worms at D1 were examined from microscopic photos. *t* test; *** $P < 0.001$. (E) *akt-1RNAi* increases the life span of *mir-84;mir-241;glp-1* and *glp-1* to the same absolute extent. A.U., arbitrary units; n, number of animals.



lives than *glp-1;daf-16RNAi* animals. Instead, *mir-84;mir-241* deletion does not further reduce life span of *glp-1* upon *daf-16RNAi* (Fig. 2C), suggesting that the miRNAs and *daf-16* work in the same pathway. To test this hypothesis, we examined the effect of the miRNAs on DAF-16 localization and activity. An *mir-84;mir-241* deficiency modestly diminished DAF-16::green fluorescent protein (GFP) nuclear localization, with little effect on overall expression levels (fig. S4, A to C). Consistent with a role in regulating DAF-16 activity via DA/DAF-12 signaling, miRNA mutation significantly reduced expression levels of six genes commonly regulated by DAF-12 and DAF-16 (Fig. 2D and fig. S5, A and B) (7). *mir-84;mir-241* also affected other DAF-16 targets, including superoxide dismutase *sod-3* and the lipase *lipl-4* (fig. S5, B and C) required for gonadal longevity (14). On *daf-16RNAi*, *mir-84;mir-241* deletion did not further decrease *daf-16*/FOXO-regulated genes (fig. S5D), suggesting that the miRNAs control these genes' expression through *daf-16*/FOXO, but not another transcription factor. By contrast, the miRNAs had little influence on the expression of *daf-12*-regulated genes *fard-1* and *cdr-6* (fig. S6, A to C) (7), nor did they influence the transcriptional output of other major regulators of gonadal longevity, including the PHA-4/FOXA targets *lgg-1* and *unc-51*, which are involved in autophagy (15), and the NHR-80 target *fat-6*, which is involved in fatty acid desaturation (fig. S6, D to F)

(13). These results reveal that *mir-84* and *mir-241* specifically stimulate DAF-16 localization and transcriptional activity.

MicroRNAs down-regulate gene expression by binding to the 3' untranslated region (UTR) of target mRNAs and reducing stability and translation. To explore targets of *mir-84;mir-241* and how they stimulate DAF-16/FOXO activity, we searched for DAF-16/FOXO inhibitors with potential *mir-84;mir-241* binding sites in their 3'UTRs, using the bioinformatic algorithm mirWIP (16). Among predicted targets were components of IR signaling, including the PDK/AKT kinase cascade, which inhibits DAF-16/FOXO (17).

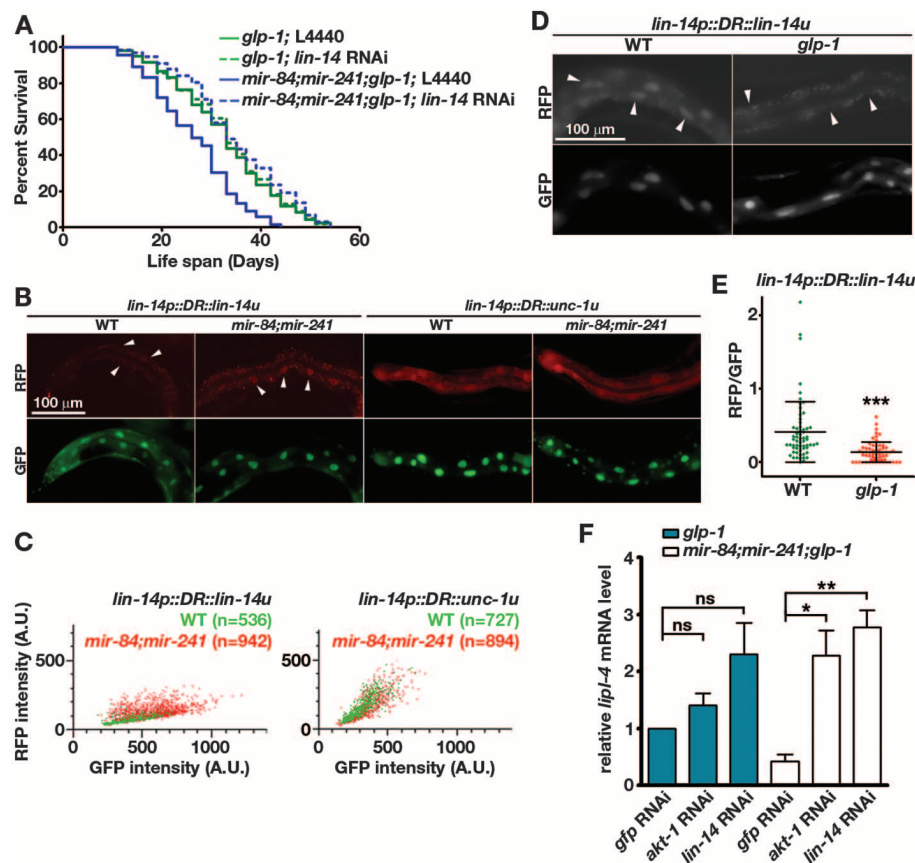
Consistent with miRNA-mediated inhibition, *mir-84* and *mir-241* significantly repressed luciferase reporters containing the 3'UTRs of *akt-1* and *pdk-1* in cell culture (fig. S7, A, G, and H). In *C. elegans*, *akt-1*-3'UTR dual reporter (DR) constructs were up-regulated in *mir-84;mir-241* double mutants relative to WT animals, whereas a *unc-1*-3'UTR-DR control reporter remained unchanged (Fig. 3, A and B, and fig. S7C), indicating an inhibition of the *akt-1* 3'UTR by *mir-84* and *mir-241* in vivo. Moreover, the *akt-1*-3'UTR-DR construct was down-regulated 40 to 50% in *glp-1* animals as compared with WT animals (Fig. 3, C and D). In contrast, a *pdk-1*-3'UTR-DR construct was not down-regulated in *glp-1* animals (fig. S7I). These results indicate that

mir-84;mir-241 down-regulates *akt-1* through its 3'UTR, in response to gonadal signals.

When we examined functional interactions between *akt-1* and the miRNAs, we observed that *akt-1RNAi* extended the life span of WT and *glp-1* mutants by 27 and 23%, respectively (Fig. 3E and table S1), consistent with previous results (18). Notably, *akt-1RNAi* enhanced longevity in *mir-84;mir-241;glp-1* triple mutants to the same absolute extent as in *glp-1* (Fig. 3E), suggesting that *akt-1* knockdown bypasses the requirement for the miRNAs. Correlatively, *akt-1RNAi* restored expression of *daf-16* target genes *sod-3* and *lipl-4* in *mir-84;mir-241;glp-1* animals (Fig. 4F and fig. S7F). These results argue that *akt-1* acts in the gonadal pathway, where the miRNAs normally antagonize *akt-1* to promote longevity, as well as independently through canonical insulin/IGF signaling (18). Because *daf-2*/IR and gonadal longevity are additive, *akt-1* could be regulated by inputs other than *daf-2* upon germline ablation and may serve as a general regulator of *daf-16*/FOXO.

The *let-7* family of miRNAs targets several genes in the heterochronic pathway, a circuit that controls larval developmental timing (19). These include genes encoding the zinc-finger protein HBL-1/hunchback and the ring-finger protein LIN-41/trim71. By mirWIP, other heterochronic genes, including those encoding nuclear protein LIN-14 and the *let-7* binding protein LIN-28 are

Fig. 4. The *lin-14* heterochronic locus is a miRNA target regulating gonadal longevity. (A) *lin-14RNAi* restores longevity to *mir-84;mir-241;glp-1*. (B and C) DR construct with the 3'UTR of *lin-14* but not of *unc-1* is up-regulated in *mir-84;mir-241* animals compared with WT animals. Arrowheads denote intestinal nuclei. (D and E) *lin-14*-3'UTR-DR is down-regulated in *glp-1*. Arrowheads denote intestinal nuclei. For each genotype, 60 intestinal cells from 20 worms were analyzed. *t* test; ****P* < 0.001. (F) *lin-14RNAi* or *akt-1RNAi* increases *lipl-4* expression in *mir-84;mir-241;glp-1*. ANOVA; **P* < 0.05, ***P* < 0.01.



predicted targets. If these genes are miRNA targets in the gonadal pathway, then their down-regulation should restore longevity to *mir-84;mir-241;gfp-1* triple mutants. RNAi treatment from L4 onwards revealed that only *lin-14RNAi* restored life-span extension to the triple mutants (Fig. 4A and table S1).

lin-14 is an intriguing candidate because Slack had previously shown that *lin-14* loss of function extends life span in a *daf-16/FOXO*-dependent manner, and *lin-14* gain-of-function mutations shorten life span (20). During development *lin-14* governs L1-L2 transitions, but its context in aging is unclear. We found that *lin-14RNAi* extended life span in WT animals as reported, but it did not further extend the life of *gfp-1* mutants (Fig. 4A). To examine its relationship with *daf-16/FOXO*, we tested whether *lin-14* knockdown influenced *daf-16* target gene expression. Similar to aging experiments in which longevity was restored, *lin-14RNAi* also significantly restored *daf-16* expression of *sod-3* and *lipf-4* to *mir-84;mir-241;gfp-1* (Fig. 4F and fig. S7F). Altogether, these observations suggest that the miRNAs down-regulate *lin-14* and promote longevity via *daf-16*.

To test this hypothesis, we examined regulation of *lin-14* by *mir-84;mir-241*. As above, a luciferase reporter with the *lin-14*-3'UTR was down-regulated by miRNAs (fig. S7B). Similarly, a *lin-14*-3'UTR-DR construct was up-regulated in *mir-84;mir-241* double mutants relative to WT, whereas the *unc-1*-3'UTR-DR controls were unchanged (Fig. 4, B and C). Consistent with a role in the gonadal pathway, the *lin-14*-3'UTR-DR construct and full-length *lin-14::gfp* were down-regulated in intestinal nuclei of germline-less animals relative to gonad-intact controls (Fig. 4, D and E, and fig. S7, D and E). Collectively, these results reveal that *lin-14*, a core component of the developmental clock, functions in the gonadal longevity circuit, where it is down-regulated by miRNAs upon germline removal.

In this work, we show that components of an early life developmental timing switch (i.e., the steroid receptor DAF-12; its ligands; its target miRNAs of the *let-7* family; and LIN-14, the miRNAs' target) are used to regulate adult life span in response to signals from the gonad. We propose a model in which they work as part of a hormone-regulated switch between reproductive and survival modes at larval to adult stage commitments (fig. S8). When GSC proliferation is prevented, unknown signals up-regulate *daf-36* and DA production by the L4/young adult stage, subsequently activating DAF-12 and its miRNA targets, *mir-84* and *mir-241*. In turn, these miRNAs down-regulate *akt-1*, *lin-14*, and possibly other targets, which stimulate DAF-16/FOXO transcriptional activity, extending survival and life span. Because miRNA deletion does not fully abolish DAF-16 activity, other signals from either gonad or DAF-12 may also prompt gonadal longevity. Conversely, when GSC proliferation ensues, DA signaling is down-regulated, miRNA

expression is low, and *lin-14* and *akt-1* expression are high, resulting in normal life span. This switch could provide a critical link between development and longevity, serving as a checkpoint monitoring the state of the germ line. For example, germline absence could mimic endogenous stress signals induced by germline quiescence or proliferative arrest in response to nutrient deprivation, infection, or damage. As components of a developmental timer, the hormone-miRNA axis could ensure coordinate metabolism, maturation, and the relative timing of events between the reproductive system and the soma, with ultimate effects on life span. It will be interesting to dissect the interaction of other miRNAs implicated in longevity with this axis (20, 21). Furthermore, these findings extend the role of *let-7* family members beyond developmental timing and differentiation to the regulation of insulin/IGF signaling and metabolism, similar to recent studies in mammals (22). Because *let-7* family members and other components of this circuitry are evolutionarily conserved, it will be interesting to see if similar pathways affect longevity in vertebrates.

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Supplementary Materials

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Hox Genes Regulate Digit Patterning by Controlling the Wavelength of a Turing-Type Mechanism

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The formation of repetitive structures (such as stripes) in nature is often consistent with a reaction-diffusion mechanism, or Turing model, of self-organizing systems. We used mouse genetics to analyze how digit patterning (an iterative digit/nondigit pattern) is generated. We showed that the progressive reduction in *Hoxa13* and *Hoxd11-Hoxd13* genes (hereafter referred to as distal *Hox* genes) from the *Gli3*-null background results in progressively more severe polydactyly, displaying thinner and densely packed digits. Combined with computer modeling, our results argue for a Turing-type mechanism underlying digit patterning, in which the dose of distal *Hox* genes modulates the digit period or wavelength. The phenotypic similarity with fish-fin endoskeleton patterns suggests that the pentadactyl state has been achieved through modification of an ancestral Turing-type mechanism.

Digit patterning has commonly been interpreted in the context of a morphogen gradient model (1, 2). The proposed

morphogen Sonic hedgehog (Shh) emanates from the zone of polarizing activity (a cluster of mesodermal cells in the posterior border of

the limb bud) and establishes a gradient with maximum levels posteriorly. Gli3 is the major mediator of Shh signaling in limb development and a genetic cause of polydactyly (2). Because Shh prevents the processing of Gli3 to its repressor form (Gli3R), the Shh gradient is translated into an inverse gradient of Gli3R (3, 4). The surprising finding that mouse *Gli3* and *Shh;Gli3* null mutants display identical polydactylous limb phenotypes demonstrates that an iterative series of digits can form in the absence of Shh (4, 5). Rather than supporting a gradient model, this observation is consistent with a Turing-type model for digit

patterning (6–11) in which dynamic interactions between activator and inhibitor molecules determine the wavelength of the specific pattern and produce periodic patterns of spots or stripes. This pattern has been hypothesized to act as a molecular prepattern for chondrogenesis. According to one of the specific predictions of the model, the digit period or wavelength, defined as the combined thickness of both digit and interdigital region, should be subject to modulation by perturbing the correct parameter of the gene network. This should lead to autopods with digits varying in thickness and number, which has never been clearly observed to date.

Although the core molecules of a self-organizing mechanism remain unknown, potential candidates for molecular modulators of the system include the *Hox* genes (10, 12). Distal *Hoxa* and *Hoxd* genes have a well-documented impact on digit number (13), though their specific role remains unclear, possibly due to their various interactions with the Shh-Gli3 pathway. These interactions include the mutual transcriptional regulation between *Hox* genes and *Shh* and the binding of Hoxd12 to Gli3R, resulting in a blockage of Gli3R repressor activity (14–16). In general, gain- and loss-of-function experiments suggest a positive relation between *Hox* genes and digit number (14, 17–22), which is also indicated by the ectopic anterior up-regulation of distal *Hoxd* genes in the *Gli3*-dependent polydactyly (4, 5). However, we showed that the combined deletion of *Hoxd11–13* and *Gli3* ex-

acerbated the *Gli3* polydactyly (23), suggesting instead a negative relation between distal *Hoxd* genes and digit number.

Hoxd11–13;Gli3 mutants displayed a gain of *Hoxa13* expression, similar to *Gli3* mutants (23). To address the relation between *Hoxa13* and digit number, we generated double *Hoxa13;Gli3* mutants (see supplementary materials and methods). At embryonic day 12.5 (E12.5), *Sox9* expression marked the five digital chondrogenic condensations in control autopods and revealed the delay in differentiation in the anterior mesoderm and the polydactyly typical of the *Gli3* deficiency (Fig. 1A) (24). *Hoxa13*^{+/+}*Gli3*^{Xtj/Xtj} showed seven to eight digital condensations in the posterior mesoderm plus a diffuse *Sox9* expression in the anterior mesoderm, which likely corresponded to presumptive digital condensations (Fig. 1A). Even though the number of digits could not be precisely determined, *Sox9* expression suggested an increase in digit number in *Hoxa13*^{+/+}*Gli3*^{Xtj/Xtj} compared with *Gli3*^{Xtj/Xtj} mutants, supporting a negative effect of *Hoxa13* on digit number, similar to distal *Hoxd* genes (23).

Hoxa13^{+/+}*Gli3*^{Xtj/Xtj} limbs also displayed reduced digit wavelength and digit bifurcations (Fig. 1A). To quantify both features, we analyzed curved anterior posterior (AP) profiles of *Sox9* expression at four equidistant positions along the proximal distal (PD) axis of the digit region of the *Hoxa13;Gli3* mutants shown in Fig. 1A (Fig. 1B and supplementary materials). We measured the average digit period and AP length of each profile (shown in

Fig. 1. (A) Expression of *Sox9* in E12.5 limbs of the *Hoxa13;Gli3* allelic series. Note the delayed differentiation in the anterior mesoderm in the absence of *Gli3*. The curved white and yellow lines show the AP profiles used for the analysis of *Sox9*. The red arrowhead points to a digit bifurcation. WT, wild type. (B) *Sox9* staining intensity along the yellow profile indicated by the curved arrow. AP length and the period of each digit (from minimum to minimum) are measured and shown for *Hoxa13*^{+/+}*Gli3*^{Xtj/Xtj}. (C) Chart showing the average digit periods versus AP lengths for each profile and limb. A linear relation is observed in controls and in the *Gli3*^{Xtj/Xtj} background for either the normal or heterozygous dose of *Hoxa13*, whereas a flatter relation that correlates with bifurcations (red arrowhead) is observed in the *Hoxa13*^{+/+}*Gli3*^{Xtj/Xtj} limbs (red line). The curved arrow marks the yellow point corresponding to the profile in (B). (D and E) Two simulations of the reaction-diffusion model inside an E12.5 *Gli3* mutant limb shape. (D) The activator concentration obtained in the simulation with a uniform modulation of wavelength ω (shown in the graph) shows digit bifurcation (red arrowhead) similar to the *Hoxa13*^{+/+}*Gli3*^{Xtj/Xtj} mutants. (E) The simulation result when wavelength is modulated according to a suitable PD gradient (in this case, a 2D gradient of simulated FGF signaling activity) avoids bifurcations, because the wavelength increases with increasing AP length. Limbs shown in all figures are forelimbs with distal to the right and anterior to the top.

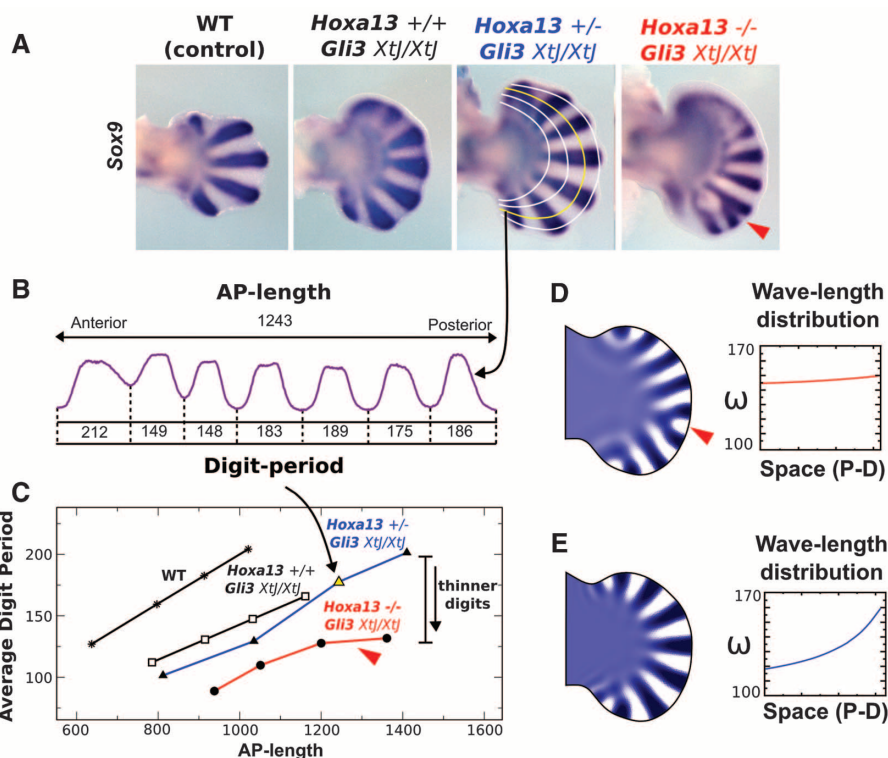


Fig. 1B for $Hoxa13^{+/-};Gli3^{XtJ/XtJ}$) and plotted results (Fig. 1C). In control, $Hoxa13^{+/-};Gli3^{XtJ/XtJ}$, and $Hoxa13^{+/-};Gli3^{XtJ/XtJ}$ mutants, the average

digit period increased along the PD axis, whereas the ratio between the average digit period and the AP length was constant, suggesting that the

wavelength of a Turing-type mechanism was scaled along the PD axis to maintain a constant number of digits. However, this was not true for

Fig. 2. Representative skeletal phenotypes of newborns of the $Hoxa13;Hoxd11-13;Gli3$ allelic series. Digit number (indicated for the $Gli3^{XtJ/XtJ}$ condition) increases as distal Hox dose is reduced. When only one functional copy of $Hoxa13$ remains (right column), the tip of the digits is connected by a continuous band of ossified (red) and cartilaginous (blue) tissue rimming the distal border of the limb and becoming more conspicuous as $Gli3$ copies are removed.

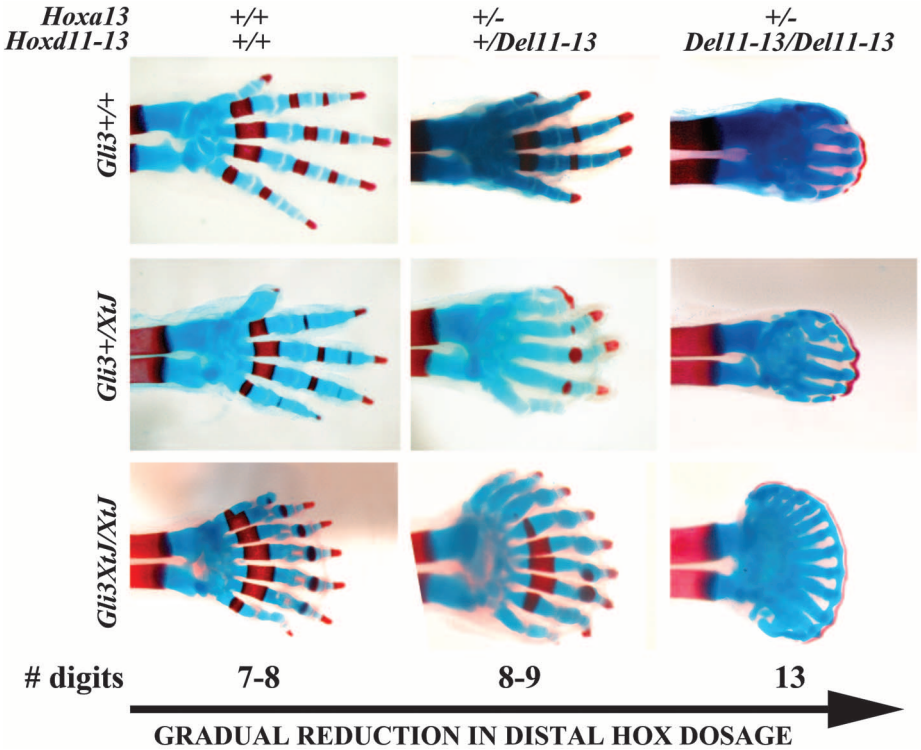
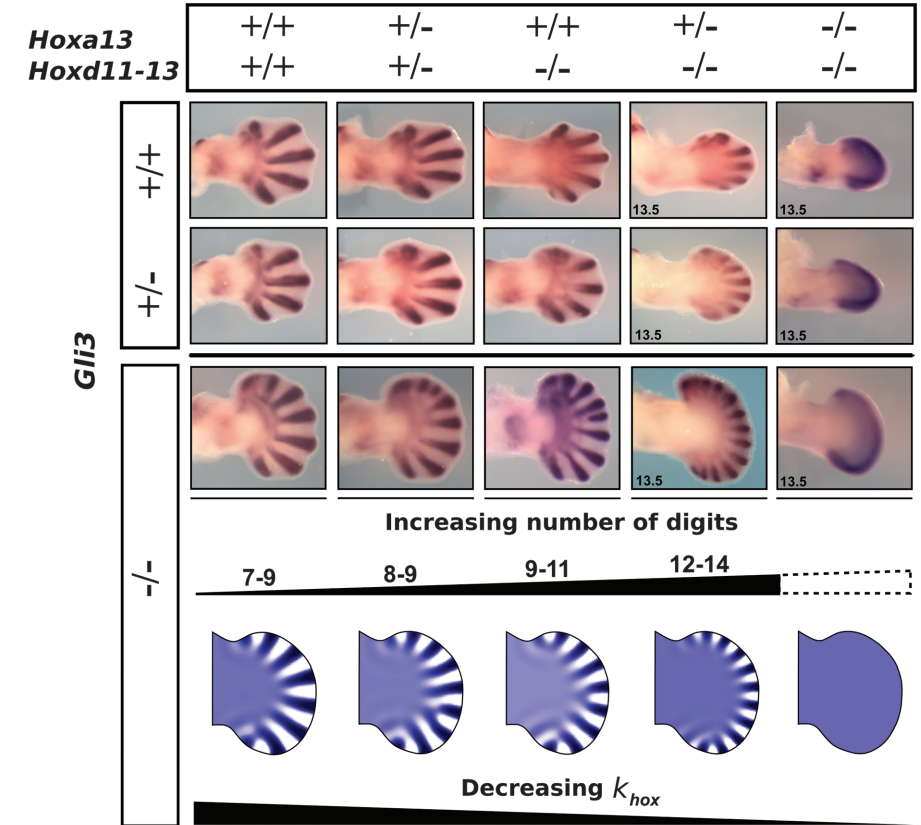


Fig. 3. The phenotypes of triple mutants can be replicated by the Turing model. (Top) The first three rows show $Sox9$ expression at E12.5 and E13.5 for different combinations of the triple $Hoxa13;Hoxd11-13;Gli3$ allelic series. As more Hox are removed, the general trend shows an increase in digit number and a decrease in digit thickness. The trend is most strongly evident in the complete absence of $Gli3$ (third row). (Bottom) A similar behavior is shown by the reaction-diffusion simulations, where a decrease of the PD gradient used to modulate wavelength is correlated with reduced Hox dose (k_{Hox}). Additionally, the model predicts a narrower digital region along the PD axis, which eventually shrinks to zero, and no pattern is formed.



the *Hoxa13*^{-/-}; *Gli3*^{Xtj/Xtj} limbs, where the average digit period flattened off distally, and, in agreement with a Turing-type mechanism, digit bifurcations occurred as the AP length increased (Fig. 1A). Our quantification suggested that normal digit patterning involves an effective PD increase of the digit wavelength, which we hypothesized must be carefully controlled if bifurcations are to be avoided.

To test this hypothesis, we built a two-dimensional (2D) finite-element model based

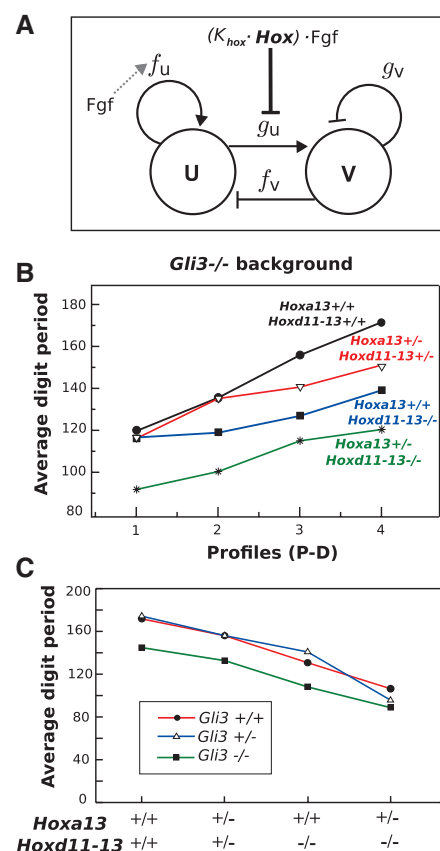


Fig. 4. (A) Schematic representation of the network of a general activator-inhibitor Turing model. The four reaction kinetic parameters are shown: f_u , f_v , g_u , and g_v . Fgf promotes a PD-graded distribution of the parameter f_u to drive stripe orientation (gray dashed arrow). Hox and Fgf inhibit the parameter g_u to increase the wavelength in a PD-graded manner (bold line). U, activator; V, inhibitor. (B) Graphs of the average digit period of the triple mutants with *Gli3*^{-/-} background at four equidistant positions along the PD axis of the digital region. With the exception of *Hoxa13*^{+/-}; *Hoxd11-13*^{-/-}; *Gli3*^{Xtj/Xtj}, a clear trend is observed: The PD gradient of wavelength is generally shallower as distal *Hox* genes are removed. (C) Graphs of average digit period (wavelength) versus distal *Hox* gene dose in the three different *Gli3* backgrounds. A smooth positive correlation between *Hox* gene dose and wavelength is observed in all three cases.

on the shape of the *Gli3*^{Xtj/Xtj} handplate at E12.5 and simulated a reaction-diffusion system by scaling the wavelength in either a uniform (remaining constant) or a graded manner along the PD axis of the digital region (Fig. 1, D and E, and supplementary text). We used a generic activator-inhibitor reaction-diffusion system, employing the minimal conditions to satisfy Turing instability (see supplementary text) (25). We determined whether a single reaction parameter of the model could be an effective target for wavelength modulation (figs. S4 and S5) and chose the effect of activator on inhibitor production (g_u) as the most suitable (see supplementary text). Our simulations showed that digit bifurcations occurred in the uniform case (red arrowhead Fig. 1D), whereas no bifurcations were shown when we used graded wavelength scaling (Fig. 1E), reproducing the observed mutant patterns. Our model suggests that, in the absence of *Gli3*, the genetic reduction of *Hoxa13* produces a global reduction in wavelength (ω), thereby increasing digit number, but also causes a shallower PD gradient of ω , which explains the observed digit bifurcations. Because there is no evidence for a PD-graded

Hox expression, the simplest interpretation of the model is that ω is modulated by both the *Hoxa13* gene (explaining the global reduction in wavelength) and fibroblast growth factor (FGF) signaling (explaining the PD gradient), possibly by co-regulating the same target genes (see supplementary text).

Because *Hoxa13*^{-/-}; *Gli3*^{Xtj/Xtj} double mutants display ectopic anterior expression of *Hoxd12* and *Hoxd13*, similar to *Gli3* mutants (fig. S1), it remained possible that the increased polydactyly was due to the gain of *Hoxd12* and *Hoxd13*. To challenge such functional compensation and test whether the cumulative sum of distal functional *Hox* genes controls digit number by modulating the digit period, we generated triple mutants carrying the *Hoxa13*^{-/-}, *Hoxd11-13*^{-/-}, and *Gli3*^{Xtj} alleles (Fig. 2). Skeletal preparations of neonates of the triple-mutant allelic series showed a variety of patterning defects, including syndactyly (fused digits), brachydactyly (shortened digits), absence of joints, ventral bending of digits, and delayed ossification. Most salient, however, was the clear trend toward increased digit number as progressively more alleles of distal *Hox* genes were removed in the

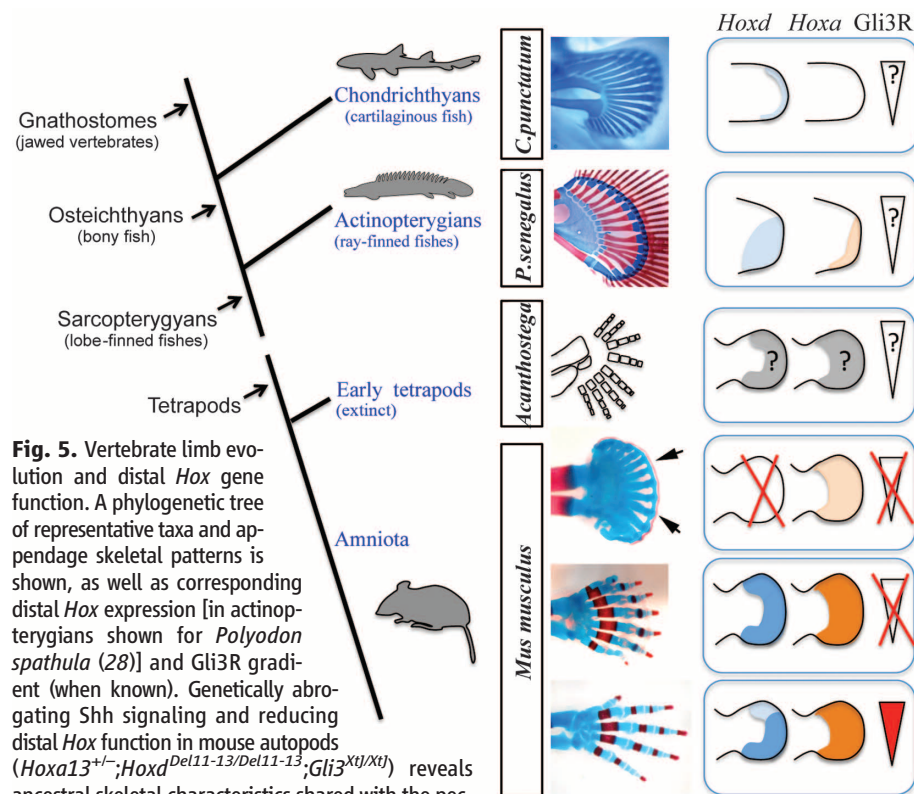


Fig. 5. Vertebrate limb evolution and distal *Hox* gene function. A phylogenetic tree of representative taxa and appendage skeletal patterns is shown, as well as corresponding distal *Hox* expression [in actinopterygians shown for *Polyodon spathula* (28)] and *Gli3R* gradient (when known). Genetically abrogating *Shh* signaling and reducing distal *Hox* function in mouse autopods (*Hoxa13*^{+/-}; *Hoxd11-13*^{-/-}; *Gli3*^{Xtj/Xtj}) reveals ancestral skeletal characteristics shared with the pectoral fins of sharks (*Chiloscyllium punctatum*) and primitive ray-finned fishes (*Polypterus senegalus*): numerous, densely packed, and iterative elements, with a distal cartilaginous band corresponding to the distal radials of fish fins (arrows). The periodic pattern of skeletal elements evident in fins and mutant limbs strongly suggests that a self-organizing Turing-type mechanism of chondrogenesis is deeply conserved in vertebrate phylogeny. Our results further indicate that distal *Hox* gene dose regulates the number and spacing of skeletal elements formed, implicating distal *Hox* gene regulatory networks as critical drivers of the evolution of the pentadactyl limb.

absence of *Gli3* (Fig. 2). The number of digits increased from 7 to 9 (typical of *Gli3^{XtJ/XtJ}*) to 8 to 9 when one functional allele of both *Hoxa13* and *Hoxd11-13* was removed; further, to 9 to 11 when both *Hoxd11-13* alleles were removed (23); and, finally, to at least 12 to 14 digits when only one copy of *Hoxa13* remained (*Hoxa13^{+/-};Hoxd11-13/Del11-13;Gli3^{XtJ/XtJ}*) (Fig. 2). The midgestational lethality of *Hoxa13* homozygous mutants precluded their analysis at this stage. The increase in digit number as distal *Hox* genes were removed did not rely on increased AP handplate size, as in *Gli3* single mutants (26), but rather on a reduced wavelength as digits were thinner and had narrower gaps between them, while remaining regularly spaced (Figs. 1A and 2). These phenotypes cannot be explained by a model of positional information based on a morphogen gradient.

To compare the principal phenotypes of the triple allelic series with our computational model, we analyzed *Sox9* expression at E12.5, when the condensations are being laid down and the Turing mechanism should be operative. Quantification of these mutants confirmed that there was no strict correlation between the progressive increase in digit number and handplate size, which instead coincided with thinner, more densely packed digits (Fig. 3). In our computer model, both *Hox* levels and FGF signaling contribute to wavelength modulation (Fig. 4A). Thus, by progressively reducing the global contribution from the *Hox* genes (k_{Hox}), the resulting ω gradient became lower, but also shallower (fig. S6), reflecting the shallower wavelength gradients quantified from the mutants (Fig. 4B). In this way, the model was able to reproduce the observed smooth series of *Sox9* phenotypes in the *Gli3^{XtJ/XtJ}* background—both the increased number of digits and the greater tendency for bifurcations (Fig. 3 and supplementary text). Although the observed reduction in wavelength was strongest in the absence of *Gli3*, the same trend was clearly apparent in the *Gli3^{+/-}* and *Gli3^{+/-}XtJ* backgrounds (Fig. 4C and supplementary materials). In these cases, a reduced wavelength does not always produce a higher number of digits, because the AP width also decreases.

Our study highlighted a developmental delay in the appearance of the *Sox9* pattern (fig. S2) and a reduction in the PD width of the digit-forming region (fig. S3). Theoretical analysis revealed that both features are naturally predicted by modulating g_u in the model. The delayed patterning also supports the possible role of FGF signaling as a Turing modulator, as FGF4 treatment of micromass-cultured mesenchyme from limb buds sped up the appearance of the pattern (25). The simulated PD gradient of FGF signaling thus translates into a gradient of patterning speed, and further theoretical analysis revealed that this naturally predicts the progressive PD narrowing of the digital zone (fig. S6 and supplementary test). Below a certain

Hox dose, the digital zone disappears entirely. Indeed, in triple mutants, no distinct digital condensations were scored, even at E13.5 (Fig. 3 and fig. S6).

In conclusion, our combination of genetics, quantitative analysis, and computer modeling reveals a missing piece of evidence for a Turing-type mechanism in digit patterning. Whereas numerous previous mutants have shown an abnormal digit number, evidence was lacking for a parameter that could smoothly tune digit wavelength. Our discovery and analysis of the smooth correlation between distal *Hox* gene number and digit wavelength provides this evidence. Additionally, the link between wavelength deregulation and the appearance of digit bifurcations also strongly supports the role of distal *Hox* genes as wavelength modulators of an intrinsic self-organizing Turing-type mechanism responsible for digit patterning. Our model predicts that overexpression of *Hox* genes should increase the digit wavelength, although this may require careful temporal examination at the time the Turing mechanism is operating and may have a subtle effect if *Hox* genes are normally expressed at saturating levels (22). The model makes no prediction about the temporal sequence of digit condensations along the AP axis. Also, the probability of bifurcations may be reduced in more extreme mutants (such as *Hoxa13^{+/-};Hoxd^{Del11-13/Del11-13};Gli3^{XtJ/XtJ}*) due to the narrower digital region. Additionally, our analysis suggests a role for *Gli3* in tuning the wavelength, but this observation is not as notable as the smooth trend seen in our distal *Hox* allelic series (Figs. 3 and 4C and supplementary materials).

Our results also permit a reassessment of distal *Hox* gene function in one of the most important vertebrate innovations: the fin-to-limb transition. The emerging consensus suggests that the genetic toolkit patterning fins and limbs is largely conserved, and the evolution of digits was driven by accumulated regulatory changes controlling the spatial and temporal deployment of that common toolkit (27–32). The reduction of the distal *Hox* gene number in the absence of *Gli3*, which renders Shh signaling irrelevant, resulted in mouse digits losing defining characteristics (pentadactyl constraint and segmented morphologies) and exhibiting patterns reminiscent of the endoskeleton patterns in chondrichthyan and basal actinopterygian fins (numerous, iterative, densely packed, infrequently segmented elements) (Fig. 5). Thus, our data provide evidence that an ancestral Turing-like mechanism patterning fins has been conserved in tetrapods and modified by the implementation of regulatory changes in the evolution of digits. In particular, our data suggest that the equilibrium resulting from the cross-regulation between Shh-Gli3 and distal *Hox* genes may have led to the stabilization of the pentadactyl state more than 360 million years ago.

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Supplementary Materials

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Arthropod Diversity in a Tropical Forest

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Most eukaryotic organisms are arthropods. Yet, their diversity in rich terrestrial ecosystems is still unknown. Here we produce tangible estimates of the total species richness of arthropods in a tropical rainforest. Using a comprehensive range of structured protocols, we sampled the phylogenetic breadth of arthropod taxa from the soil to the forest canopy in the San Lorenzo forest, Panama. We collected 6144 arthropod species from 0.48 hectare and extrapolated total species richness to larger areas on the basis of competing models. The whole 6000-hectare forest reserve most likely sustains 25,000 arthropod species. Notably, just 1 hectare of rainforest yields >60% of the arthropod biodiversity held in the wider landscape. Models based on plant diversity fitted the accumulated species richness of both herbivore and nonherbivore taxa exceptionally well. This lends credence to global estimates of arthropod biodiversity developed from plant models.

Most eukaryote species are terrestrial arthropods (1), and most terrestrial arthropods occur in tropical rainforests (2). However, considerably greater sampling effort is required in tropical arthropod surveys to yield realistic estimates of global species richness (3–7). A basic hindrance to estimating global biodiversity lies in a lack of empirical data that establish local biodiversity, which can be scaled up to achieve a global estimate.

Although many studies reported species richness for selected groups of well-studied insect taxa, no satisfactory estimate of total arthropod species richness exists for a single tropical rainforest location to date.

The unstructured collection and small-scale survey of tropical arthropods cannot yield convincing estimates of total species richness at a specific forest (7–9). Most studies either target few arthropod orders or trophic guilds, or use a limited array of sampling methods, or ignore the diverse upper canopy regions of tropical forests (10–15). Moreover, sampling protocols have rarely been structured in such a way that, with increased sampling, incomplete data on local diversity (7) can be extrapolated to estimate total species richness across multiple spatial scales (16). Where such structured estimates are made, it is invariably for insect herbivores on their host plants (5). However, species accumulation rates may differ markedly for nonherbivore guilds, which include more than half of all described arthropod species (1, 17). As the degree of host specificity (effective specialization) of other guilds can be much lower than that of insect herbivores, or may be driven by different factors (18, 19), global estimates based on herbivores alone are questionable. Consequently, extensive cross-taxon surveys with structured protocols at reference sites may be the only effective approach toward estimating total arthropod species richness in tropical forests (3).

To provide a comprehensive estimate of total arthropod species richness in a tropical rainforest, we established a collaboration involving 102 researchers with expertise encompassing the full breadth of phylogenies and feeding modes present among arthropods (20). This consortium invested a total of 24,354 trap- (or person-) days sampling the San Lorenzo forest (SLPA) in Panama using structured protocols (fig. S1). We identified

129,494 arthropods representing 6144 focal species (Fig. 1 and table S1) from 0.48 ha of intensively sampled mature forest. This allowed us to extrapolate focal arthropod species richness to a larger forest area with unprecedented power, through a series of best-informed species richness estimates derived from six competing models for each of 18 focal data sets. Using taxon ratios to estimate the species richness of nonfocal taxa [see “Extrapolating results to nonfocal taxa” in materials and methods (20)], we then predicted the total species richness of the study area. We also evaluated differences in relative species accumulation rates among arthropod guilds, across spatial scales.

Although individual estimators adjusting for different aspects of sampling design offered slightly different estimates (Fig. 1B), the total species richness for the entire San Lorenzo forest (~6000 ha) was consistently quantified at between 18,000 and 44,000 species (including focal and nonfocal species). In particular, the most likely lower bound of species richness was estimated to be at least 21,833 species [95% confidence level (CL) = 18,665, 29,420; model a1 in Fig. 1B], and the biologically and statistically best-supported estimate of richness (criteria outlined in table S2) was 25,246 species (95% CL = 19,721, 33,181, model B+S in Fig. 1B). According to our estimates, a single hectare of rainforest will be inhabited by an average of 18,439 species (95% CL = 17,234, 18,575; Fig. 2B).

A relatively large proportion of the expected species richness of the forest was recovered for most of our focal taxonomic groups (Fig. 2). For example, high proportions of all ant species and of the parasitoid species targeted in our study were collected from our 12 sites, whereas fungal feeders would require more intensive sampling to achieve adequate coverage (Fig. 2). Beta diversity of all arthropods (in the broad sense of species turnover among sites) increased roughly linearly with cumulative area surveyed ($F_{1,3} = 2422.5$, $P < 0.001$). With increasing sampling effort, sample coverage [an unbiased measure of sample completeness, see (20)] was high and accumulated at significantly different rates across different arthropod orders and guilds, and across the various guilds comprised by beetles (Fig. 3). However, despite the high sample coverage values, we cannot discount the possibility that there were some vanishingly rare species that may not have been discovered with the sampling protocols used in this study.

Despite idiosyncrasies in the rate of increase in sample completeness across insects groups, the high proportion of overall species richness detected at small spatial scales (Figs. 2 and 3) has a remarkable consequence. Based on a general relationship between species numbers and area, we estimate that almost two-thirds (64%) of all species in SLPA occur in a single hectare of rainforest (Fig. 2). Our plant models predicted total arthropod richness in the San Lorenzo forest to a precision of 1% (correlation between rich-

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ness estimates provided by the plant model and best estimates: $r = 0.992$, $P < 0.0001$, $N = 18$; Fig. 1C). Notably, small discrepancies between observed arthropod species richness and estimates derived from floristic diversity appeared not to be scale-dependent (Fig. 1D). Hence, even for arthropod guilds other than herbivores, plant diversity seems a powerful predictor of species richness across areas varying in size (at least within the limits of our study design and given

the limited heterogeneity of the study area compared to larger geographical scales). Because this study targeted the full spectrum of arthropods, it offers a comprehensive test of previous estimates of species richness based only on selected guilds or taxa. Reassuringly, our well-resolved estimates of tropical arthropod species richness are of the same order of magnitude as prior estimates (table S3), adding credence to recent estimates of tropical arthropod diversity

(5, 21). Although the scope for direct comparison is limited because of regional differences in sampling effort, lowland tropical forest in Panama seems to support 2.1 to 8.4 times as many arthropod species as observed in temperate forests (table S3). While this supports the obvious truism that tropical arthropods are indeed more diverse than their temperate counterparts (22), the magnitude of that difference is far lower than many previous estimates would suggest (2).

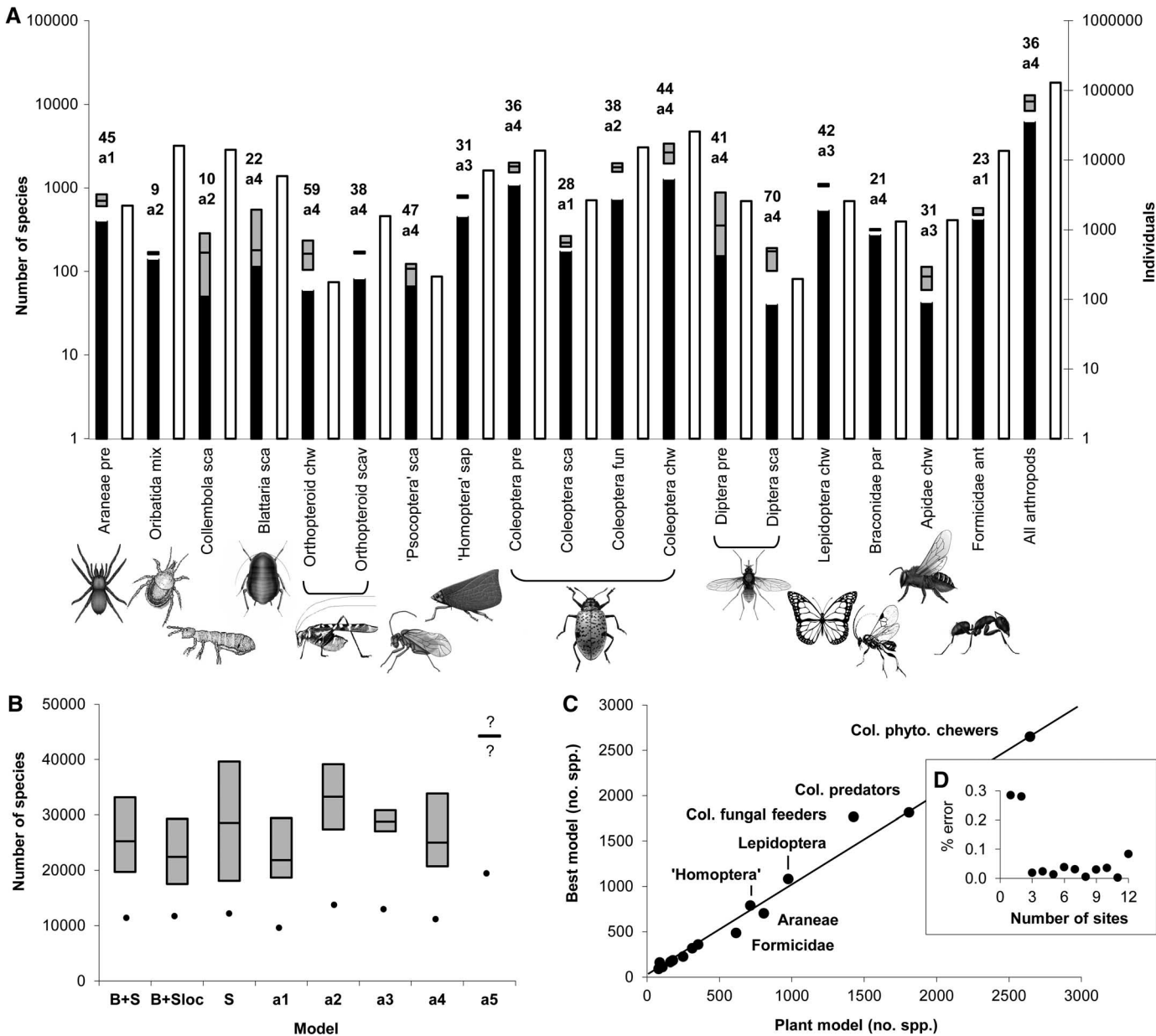


Fig. 1. Number of arthropod species estimated at SLPA (20). (A) Number of species (closed bars, log scale) and individuals (open bars, log scale) collected in 0.48 ha for each data set (three-letter guild code as in table S1) and number of species estimated in SLPA (best estimate, shaded boxes). Numbers above bars identify the best model used for calculation (a1 to a6, fig. S2) and the percentage of singletons. (B) Number of arthropod species estimated for SLPA (dots: all focal taxa; shaded boxes: focal and nonfocal taxa; table S4), as estimated by different methods: B+S: best estimates, including both biological and statistical arguments (table S2); B+Sloc: same as B+S but estimates

calculated with local instead of global ratios (fig. S3); S: best estimates, including only statistical arguments; and models a1 to a5. ?: optimization algorithms did not converge to allow calculations of CL. Our estimates are robust to even moderate to large shifts in taxon ratios (table S5). (C) Plot of the number of species estimated in SLPA with the plant model against that estimated with the best model, for each data set. Line denotes unity. (D) Plot of the percentage error between all arthropod species observed and estimated by the plant model against cumulative number of sites. Shaded boxes indicate means and 95% CL.

The implications of the results observed on a local scale are clear. For every species in the well-known vascular flora (1294 species), avifauna (306 species), and mammalian fauna (81 species) of SLPA, we estimate that there will be a minimum of 17, 71, and 270 arthropod species, respectively (based on lower bound of species richness) and most likely as high as 20, 83, and 312 arthropod species, respectively (table S3). Based on the dominance of arthropod species

in the tropical fauna (table S3), we may then argue that conservation planning for biodiversity should be largely determined by the spatial scaling of arthropod diversity. In this context, the association between the species richness of plants and arthropods detected across spatial scales suggests that conservation efforts targeted at floristically diverse sites may also serve to conserve arthropod diversity across both taxonomic lineages and trophic guilds. As arthropods are notoriously

labor-intensive to survey, such an “umbrella” approach may be an efficient way forward.

Nonetheless, our findings also suggest that large-scale, region-wide understanding of tropical arthropod richness may actually be more achievable than previously assumed. Our data indicate that a thorough sampling of 1 ha of rainforest may reveal nearly two-thirds of all arthropod species present in a much larger area (6000 ha in our case; Fig. 2B), consistent with

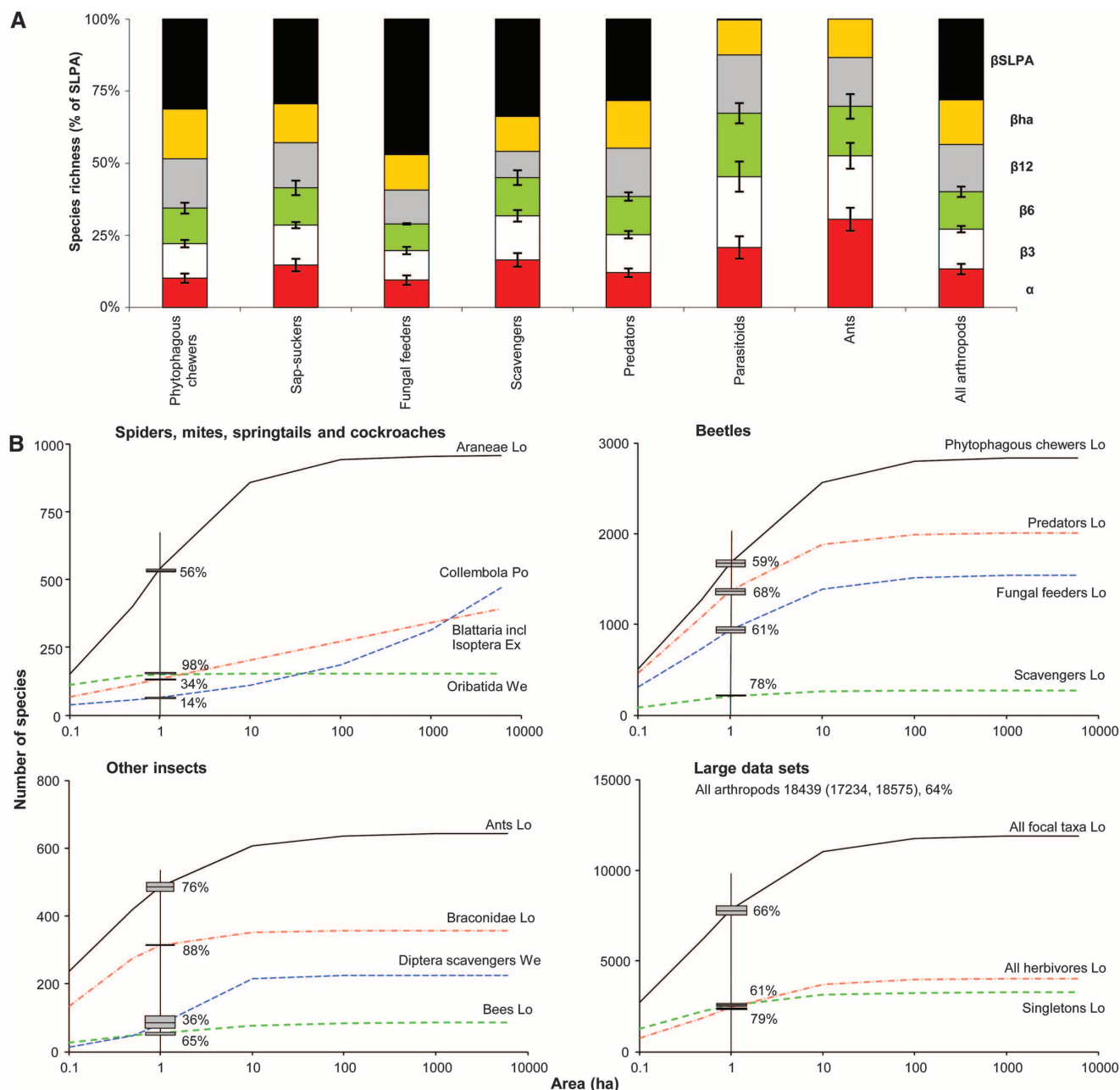


Fig. 2. Accumulation of species richness with area at SLPA (20). For all groups, a high proportion of overall species richness was detected at small spatial scales. **(A)** Partitioning of species richness within arthropod guilds at different spatial scales (α : single site of 0.04 ha; β_3 : three sites spaced apart totaling 0.12 ha; β_6 : six sites totaling 0.24 ha; β_{12} : 12 sites totaling 0.48 ha; β_{ha} : 1.0 ha; β_{SLPA} : 6000 ha; means $\pm$ SEM are shown for α , β_3 , and β_6). **(B)** Species-area models for the main arthropod groups and large

data sets (for all arthropods, including nonfocal species, values are indicated but the curve not plotted for the sake of clarity). Each curve is characterized by its function (Ex, Exponential; Lo, Lomolino; Po, Power; We, cumulative Weibull), its value for 1 ha (intersection with vertical line, shaded boxes with mean and 95% CL), and the percentage of the number of species present in 1 ha relative to the number of species estimated to occur in SLPA.

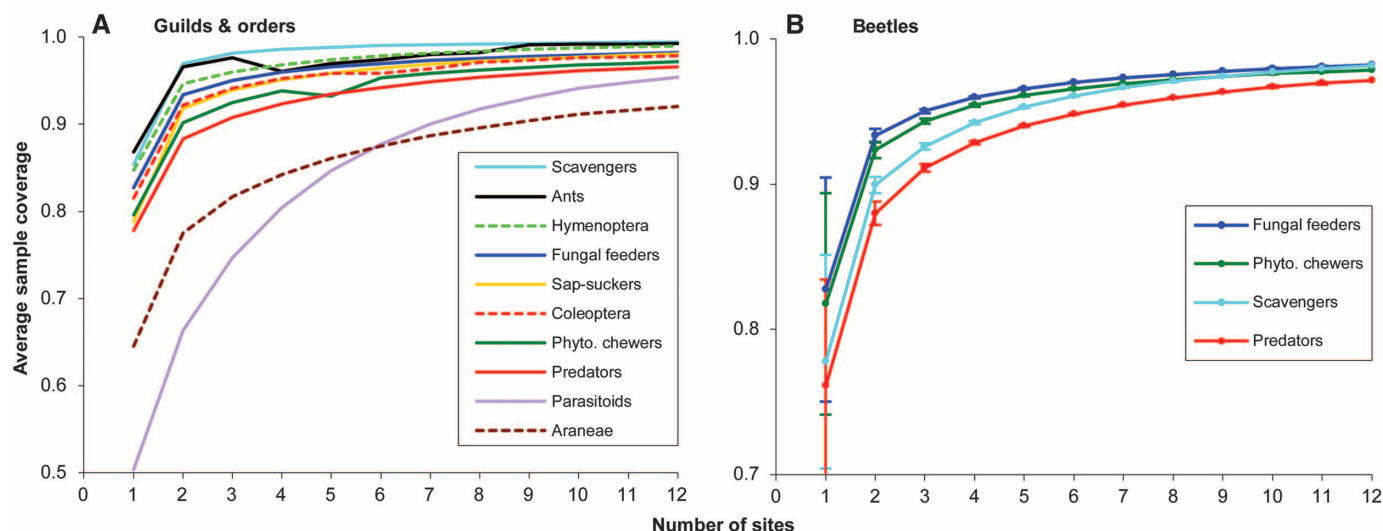


Fig. 3. Average sample coverage [$\pm$ SEM; error bars, see methods (20)] plotted against the cumulative number of sites surveyed, for the main (A) arthropod guilds and orders and (B) beetle guilds. For the sake of clarity, SEMs are omitted in (A).

reports of relatively low beta diversity of insect herbivores in tropical rainforests (23). Hence, to determine the species diversity of a tropical rainforest, the total area sampled need not be overly large—provided that the sampling design adequately covers both microhabitats and plant species. However, this does not imply that most arthropod species have self-supporting populations in small forest areas or fragments.

On a global scale, our results have implications for current estimates of total species richness, which have been weakened by the lack of knowledge regarding the strength of association between vascular plant species and nonherbivore guilds (5). Based on the close association observed here between floristic diversity and both herbivore and nonherbivore species richness, we tentatively conclude that the most recent estimate of global tropical arthropod species [6.1 million arthropod species (24)] does not require drastic correction to account for differential scaling relationships of nonherbivore taxa. The robust estimates of local arthropod diversity derived in our study thus support previous estimates of global species richness. They also show how stratified sampling designs and broad scientific cooperation may be developed to formulate efficient estimates of tropical arthropod diversity. Similar initiatives have recently been implemented in

other tropical locations around the world, using the current template as a foundation (25).

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Supplementary Materials

www.sciencemag.org/cgi/content/full/338/6113/1481/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S3
Tables S1 to S5
References (26–151)

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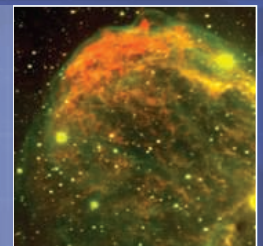
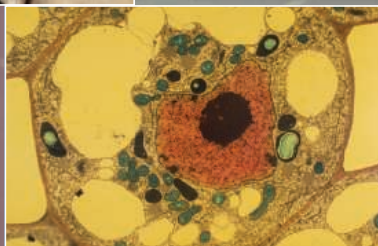
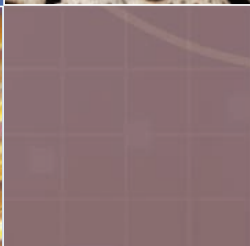
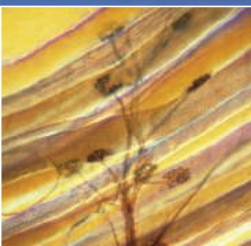
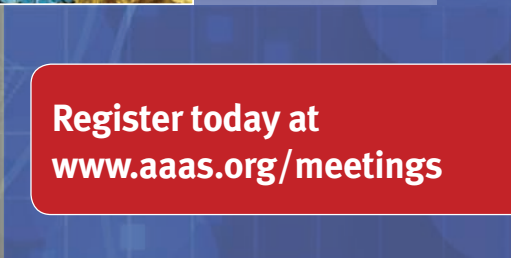
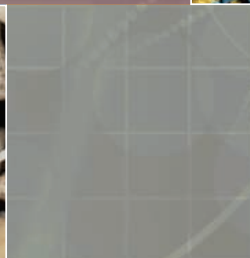
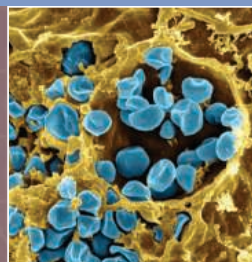
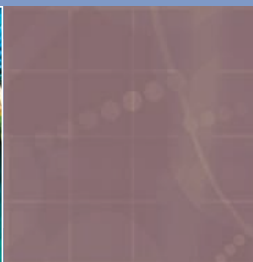
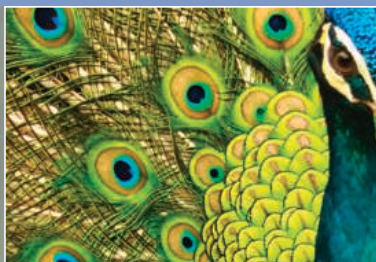
LIST OF PROTEINS

4-1BBL	Caspase-3	sFlt-1 (D3)	IL-2	MEC	sRANK
4-1BB Receptor	Caspase-6	sFlt-1 (D4)	IL-3	Mek-1	sRANKL
6 Ckine	CD4	sFlt-1 (D5)	IL-4	MIA	RANTES
ACAD8	CD14	sFlt-1 (D7)	sIL-4 Receptor	Midkine	RELM- α
ACAT2	CD22	Flt3-Ligand	IL-5	MIG / CXCL9	RELM- β
gAcrp30/Adipolean	CD40 Ligand / TRAP	sFlt-4	IL-6	MIP-1 α / CCL3	Resistin
Activin A	CD95 / sFas Ligand	sFlt-4/ Fc Chimera	sIL-6 Receptor	MIP-1 β / CCL4	RPTP β
ACY1	CD105 / Endoglin	Follistatin	IL-7	MIP-3 / CCL23	RPTP γ
ADAT1	CHIPS	FSH	IL-8 (72 a.a.)	MIP-3 α / CCL20	RPTP μ
Adiponectin	CNTF	Fractalkine/ CX3C	IL-8 (77 a.a.)	MIP-3 β / CCL19	SCF
ADRP	Collagen	G-CSF	IL-9	MIP-4 (PARC) / CCL18	SCGF- α
AITRL	CREB	α -Galactosidase A	IL-10	MIP-5 / CCL15	SCGF- β
Akt1	CTACK/CCL27	Galectin-1	IL-11	MMP-3	SDF-1 α
Alpha-Feto Protein (AFP)	CTGF	Galectin-3	IL-12	MMP-7	SDF-1 β
Alpha-Galactosidase A	CTGFL/WISP-2	Gastrointestinal CA	IL-13	MMP-13	Secretin
Angiopoietin-1 (Ang-1)	CTLA-4/Fc	GCP-2	IL-13 analog	Myostatin	SF20
Angiopoietin-2 (Ang-2)	CXCL16	GDF-3	IL-15	Nanog	SHP-2
Angiostatin K1-3	Cytokeratin 8	GDF-9	IL-16 (121 a.a.)	NAP-2	STAT1
Annexin-V	DEP-1	GDF-11	IL-16 (130 a.a.)	Neurturin	c-Src
apo-SAA	Desmopressin	GDNF	IL-17	NFAT-1	TACI
Apolipoprotein A-1	Disulfide Oxidoreductase	GLP-1	IL-17B	beta-NGF	TARC
Apolipoprotein E2	E-selectin	Glucagon	IL-17D	NOGGIN	TC-PTP
Apolipoprotein E3	ECGF	Goserelin	IL-17E	NOV	TECK
Apolipoprotein E4	EGF	GM-CSF	IL-17F	NP-1	TFF2
APRIL	Elafin/SKALP	GPBB	IL-19	NT-1/BCSF-3	TGF- α
Artemin	EMAP-II	GRO α	IL-20	NT-3	TGF- β 1
ATF2	ENA-78	GRO β	IL-22	NT-4	TGF- β 2
Aurora A	Endostatin	GRO γ	IL-31	Ocreotide	TGF- β 3
Aurora B	Enteropeptidase	GRO/MGSA	Insulin	Oncostatin M	Thymosin α 1
BAFF	Eotaxin	Growth Hormone	IP-10	Osteoprotegerin (OPG)	sTIE-1/Fc Chimera
BAFF Receptor	Eotaxin-2	Growth Hormone BP	JE	OTOR	sTIE-2/Fc Chimera
BCA-1 / BLC / CXCL13	Eotaxin-3 (TSC)	GST-p21/WAF-1	JNK2a1	Oxytocin	TL-1A
BCMA	EPHB2	HB-EGF	JNK2a2	p38- α	TNF- α
BD-1	EPHB4	HCC-1	KC / CXCL1	Parathyroid Hormone	TNF- β
BD-2	Eptifibatide	HGF	KGF	PDGF-AA	sTNFR1
BD-3	Erk-2	Histidyl-tRNA synthetase	L-asparaginase	PDGF-AB	sTNFR2
BDNF	Erythropoietin (EPO)	Histrelin	LAG-1	PDGF-BB	TPO
Bivalirudin	Exodus-2	HRG1- β 1	LALF Peptide	Persephin	TRAIL/Apo2L
BMP-2	Fas Ligand	I-309	LAR-PTP	PF-4	sTRAIL R-1 (DR4)
BMP-4	Fas Receptor	I-TAC	LC-1	PIGF-1	sTRAIL R-2 (DR5)
BMP-7	FGF-1 (acidic)	IFN- α	LBP	PIGF-2	TSH
BMP-13	FGF-2 (basic)	IFN- α A	LD-78 β	PKA α -subunit	TSLP
sBMPR-1A	FGF-4	IFN- α 2a	LDH	PKC- α	TWEAK
Brain Natriuretic Protein	FGF-5	IFN- α 2b	LEC/NCC-4	PKC- γ	TWEAK Receptor
BRAK	FGF-6	IFN- β	Leptin	Pleiotrophin	Urokinase
Breast Tumor Antigen	FGF-7/ KGF	IFN- γ	LIGHT	PLGF-1	VEGF121
C5a	FGF-8	IFN-Omega	LIX	Polymyxin B (PMB)	VEGF145
C5L2 Peptide	FGF-9	IGF-I	LKM	PRAS40	VEGF165
C-10	FGF-10	IGF-II	LL-37	PRL-1	VEGF-C
C-Reactive Protein	FGF-16	proIGF-II	Lymphotactin	PRL-2	VEGF-C I525
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Calbindin D-28K	FGF-19	IGFBP-3	MCP-1 (MCAF)	Prolactin	HB-VEGF-E
Calbindin D-29K	FGF-20	IGFBP-4	MCP-2	Protirelin	sVEGFR-1
Calmodulin	sFGFR-1 (IIIc) / Fc Chimera	IGFBP-4	MCP-3	PTHrP	sVEGFR-2
Calcitonin Acetate	sFGFR-2 (IIIc) / Fc Chimera	IGFBP-5	MCP-4	PTP1B	sVEGFR-3
Carbonic Anhydrase III	sFGFR-3 / Fc Chimera	IGFBP-6	MCP-5	PTP-IA2	WISP-1
Carcino-embryonic Antigen	sFGFR-4 / Fc Chimera	IGFBP-7	MDC (67 a.a.)	PTP-MEG2	WISP-2
Cardiotrophin-1	sFlt-1 (native)	IL-1 α	MDC (69 a.a.)	PTP-PEST	WISP-3
		IL-1 β	MDH		WNT-1

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We'll be sending you more information about this great new benefit over the coming months. In the meantime, be sure to visit nasafcu.com/AAASpackage to apply for the new AAAS Platinum Advantage Rewards or Platinum Cash Rewards credit cards. You can also take a sneak peek at the AAAS Check Card and Checks coming soon.

Sincerely,

A handwritten signature in blue ink, appearing to read 'Ian King'.

Ian King
Director of Marketing and Membership, AAAS





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Science Translational
Medicine is pleased
to announce the

AAAS MARTIN AND ROSE WACHTEL CANCER RESEARCH AWARD

This new annual award will honor an early-career cancer researcher who has performed outstanding work in the field of cancer research. Nominees must have received their PhD or MD within the last 10 years. The award winner will be invited to deliver a public lecture on his or her research and will receive a cash award of \$25,000. The award winner's Perspective will be published in *Science Translational Medicine*.

Call for Nominations

Due by Friday
February 1st, 2013

Nomination packages must include the following materials, written in English:

- » A letter describing the nominee's significant contributions to cancer research, including supporting publications. The letter should explain how the candidate's research promises to make a lasting impact on the cancer field. (Applicants may nominate themselves.)
- » A letter of support from another scientist who knows the applicant's work.
- » The nominee's curriculum vitae.
- » A Perspective (1500 words maximum, 1 figure) written by the nominee describing their research project and explaining how it advances our understanding of cancer. The research described in the Perspective must be in the field of cancer and the nominee must have performed or directed the work within the last 10 years.

Submit nominations to wachtelprize@aaas.org and put "Wachtel Award Nomination" in the subject line.

This award is funded by an endowment established through a bequest from Martin Wachtel and is presented by Science Translational Medicine.

LOCATION: Jackson
Park Health Club

ARTICLE: *An Electronic
Second Skin*

DATE: Sep 21, 7:43am

LOCATION: University
Faculty Lounge

ARTICLE: *The Visual
Impact of Gossip*

DATE: Sep 21, 4:22pm

LOCATION: Hemlock Bar

ARTICLE: *Quantum
Simulation of Frustrated
Classical Magnetism in
Triangular Optical Lattices*

DATE: Sep 21, 9:21pm

LOCATION: Gyro King

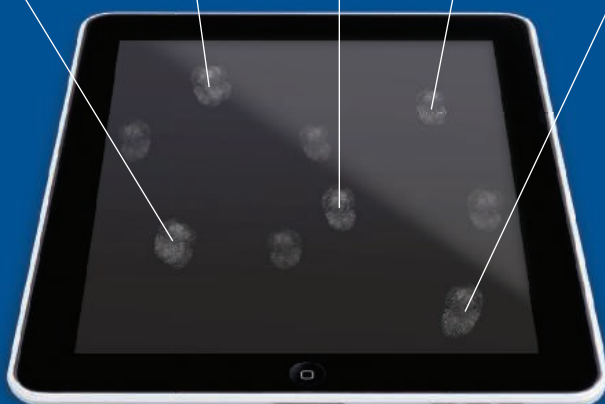
ARTICLE: *Cavemen
Craved Carbs, Too*

DATE: Sep 21, 1:13pm

LOCATION: Bed

ARTICLE: *Consciousness:
What, How and Why*

DATE: Sep 21, 10:56pm



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The Helmholtz Zentrum München, German Research Center for Environmental Health and the Faculty of Biology of LMU invite applications for a

Head of Institute and Full Professorship (W3) of Immunobiology

commencing as soon as possible.

Both institutions are seeking an outstanding leader in the field to complement the HighTech Campus Großhadern/Martinsried, one of Europe's largest life science research aggregations. Special consideration is given to scientists who have a strong basic research program and excellent track record in the field of molecular and genetic mechanisms that guide ontogenesis, differentiation and function of immune cells and their response to environmental challenge. Research on response to biotic stress and the balance between pathogen defense and tolerance to self is particularly welcome. The successful candidate is expected to participate in teaching Genetics at the Faculty of Biology.

Prerequisites for this position are a university and a doctoral degree, teaching skills at university level, excellent academic achievements and a productive and promising research program.

The future holder of this position will be appointed to LMU as a university professor (pay grade W3) under the terms of a private-law contract and will be granted leave of absence in order to lead the Institute at Helmholtz Zentrum München based on an employment agreement under private law.

The Helmholtz Zentrum München and LMU are equal opportunity employers and aim to increase the number of female faculty members. Therefore, applications from female candidates are explicitly encouraged. Both institutions support dual career couples. Disabled candidates with essentially equal qualifications will be given preference.

Information concerning the scientific scope of the position can be obtained from Prof. Wolfgang Hammerschmidt, Helmholtz Zentrum München (hammerschmidt@helmholtz-muenchen.de, phone +49 89 3187-1506) or Prof. Michael Boshart, Ludwig-Maximilians-Universität München (boshart@lmu.de, phone +49 89 2180-74600).

Applications including curriculum vitae, list of publications, a brief summary of present and future research interests, proof of teaching experience and copies of relevant documents should be sent as hard copy before **February 1, 2013** to Prof. Dr. Günther Wess, CEO and President, Helmholtz Zentrum München, Ingolstädter Landstr. 1, D-85764 Neuherberg. In addition, the form available at <http://www.biologie.uni-muenchen.de/fakultaet/organisation/dekanat/index.html> needs to be completed and send together with the application as a PDF to the E-Mail-address: dekanat19@lmu.de.

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ASSISTANT PROFESSOR/TENURE TRACK Department of Biology Genomics

California State University, Northridge invites applications for a tenure-track position in the Department of Biology. Applicants must hold a Ph.D. and have postdoctoral experience. The successful candidate shall develop a vigorous research program involving undergraduate and Master's students, seek extramural research funding, and demonstrate teaching excellence.

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Screening will begin on February 1, 2013.

Application Procedure: Please visit **website: <http://www.csun.edu/facultyaffairs/openings/sm/>**
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FACULTY POSITION IN IMMUNOLOGY Department of Pathology and Microbiology University of Nebraska Medical Center

ASSISTANT or ASSOCIATE PROFESSOR is sought focused on the immunology of infectious disease. Successful candidate will have a demonstrated track record of extramural funding or display a high potential based on publications and original concepts. The appointee will possess a strong commitment to collaborative approaches to research, and will participate in the academic mission of the department, including graduate teaching in immunology and mentoring of students and postdoctoral fellows. Resources include collaborators and programs with the NIH-funded Center for Staphylococcal Research (**website: <http://www.unmc.edu/pathology/csr/>**). Applicants must have a Ph.D., M.D., or equivalent degree, and research experience in immunology or immunology of infectious diseases. Please submit electronically a letter of interest, curriculum vitae, description of future research plans, and the names of three references to: **Immunology Search Committee Chair, Path/Micro, UNMC, Omaha, NE 68198** (e-mail: kwarchola@unmc.edu). To ensure full consideration, applications should be received by March 1, 2013, but will be accepted until the position is filled. *Individuals from diverse backgrounds are encouraged to apply.*

ASSISTANT PROFESSOR in Evolutionary Biology, Two Positions Available

The Department of Biology invites applications for two nine-month tenure-track Assistant Professor positions (50% research, 40% teaching, and 10% service) in Evolutionary Biology. Preference for both positions will be given to candidates who are employing large genomic data sets to explore compelling evolutionary questions. One position will emphasize the evolution of structure, function, or development at the level of whole organisms, physiological systems, or molecules. The other position will emphasize microevolution, such as population genetics, speciation, ecological invasion, or responses to changing environmental conditions (including climate change). See **website: <http://jobs.usu.edu>** (Req. ID 053550) for more information and to apply online. *Affirmative Action/Equal Opportunity Employer.*

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BIOCHEMISTRY

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The Saint James School of Medicine, an international medical school (**website: <http://www.sjsm.org>**), invites applications from candidates with teaching and/or research experience in any of the basic medical sciences for its Caribbean campuses. Faculty positions are currently available in Anatomy, Physiology, Pathology, Microbiology, Biochemistry, Physical Diagnosis, Clinical Correlation of Basic Sciences, and Pharmacology. Applicants must be M.D., D.O., and/or Ph.D.

Teaching experience in the U.S. system is desirable but not required. Retired persons are encouraged to apply. Attractive salary and benefits. Submit curriculum vitae to **e-mail: mjansen@mail.sjsm.org** or by mail to: **HRDS Inc., 1480 Renaissance Drive, Suite 300, Park Ridge, IL 60068.**

PROGRAM MANAGER

University South Carolina (USC) School of Medicine Department of Pathology/Microbiology/Immunology, **Requisition #5329**. Applications are invited for the position of Program Manager. Master's degree in related field and four years relevant experience, or Bachelor's degree with six years' relevant experience required. The candidate will be responsible for grant and manuscript writing, progress report preparation and obtaining approvals from regulatory boards. The University of South Carolina requires individuals to complete and online application. You may access USC Jobs Online Employment site at **website: <https://uscjobs.sc.edu>**.

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DEPARTMENT HEAD, HORTICULTURE Oregon State University

Required qualifications include a Ph.D. in a field relevant to Horticulture and a significant record of achievement in teaching, scholarship, outreach, and service. To review posting and apply, go to **website: <http://oregonstate.edu/jobs>**. Apply to **posting #0009980**. *Oregon State University is an Affirmative Action/Equal Opportunity Employer.*

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Imperial College London, Silwood Park Campus

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Up to 10 positions to be appointed within the Faculty of Natural Sciences

- Imperial College London is launching a major initiative to tackle the grand challenges and opportunities facing ecological systems and the environment.
- The initiative will appoint a large cohort of scientists in a flexible set up, from permanent academics to sabbaticals and members of collaborative working groups.
- This initiative will benefit from, and invest in, long-term field experiments both at Silwood Park and around the world, as well as in-house, high-tech laboratory facilities.
- Bringing together some of the top leaders across disciplines, the initiative will strive to ensure the future wellbeing of humanity and ecological systems in a world of global change.
- The initiative will involve groups from across the Faculties of Natural Sciences, Medicine and Engineering, the Grantham Institute for Climate Change, and the Centre for Environmental Policy.

We are seeking scientists at all levels (Lecturer/Reader/Chair) to develop innovative projects addressing challenges in areas including the following:

- Land-use change and interactions between ecosystem processes, biodiversity and human health and wellbeing; Sustainable food and water supplies; Human-environment-biosphere interactions; Environmental medicine; the response of ecosystems to a changing climate & broader environment, and their feedback on this change.
- Managing focal species in complex ecosystems; Disease, pest, invasive and/or vector biology; Ecological synthetic biology and genomics.
- Predicting, monitoring and mitigating environmental and biotic change across local to global scales; Ecosystem science, including modelling, field and laboratory experiments; Remote sensing ecology and automated monitoring systems; Ecological engineering and prediction.

The initiative will be based in the Department of Life Sciences at the Silwood Park Campus (<http://www3.imperial.ac.uk/silwoodparkcampus>), with the option of joint affiliations with other departments where applicable.

Successful applicants must have an international research profile, demonstrated by a strong portfolio of publications in top journals within the last five years, and the drive to tackle grand challenges.

For an informal discussion about the posts please contact Professor Tim Barraclough, Chair of the search committee, (t.barraclough@imperial.ac.uk, tel: +44 (0)207 594 2247).

Our preferred method of application is online via our website <http://www3.imperial.ac.uk/employment> (please select "Job Search", then enter "Ecosystems" or vacancy reference number including spaces - **NS 2012 250 JT** - into "Keywords"). Please complete and upload an application form as directed.

Alternatively, if you are unable to apply online, please contact Mrs Diana Anderson on +44 (0)207 594 2207 or e-mail: d.anderson@imperial.ac.uk to request an application form.

Closing date: 31 January 2013.

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DIRECTOR
Fermi National Accelerator Laboratory



The Board of Directors of Fermi Research Alliance, LLC (FRA) has initiated a search for a new Director of the Fermi National Accelerator Laboratory in Batavia, Illinois. The position, for a term of five years with the possibility of extension for a mutually agreed time, will be available July 1, 2013. The Search Committee welcomes applications and nominations for this position. It is recommended that applications be accompanied by curriculum vitae and other information bearing on the candidates' qualifications for the Directorship. Relevant qualifications include visionary leadership capability, internationally recognized scientific achievement, management experience and accomplishments at a national laboratory or complex research setting, and broad communications skills.

The membership of the Search Committee, its charge, and provision for submitting confidential input to the Committee are posted at <http://www.fnal.gov/pub/directorsearch>

Communications should be sent as soon as possible, preferably before **January 15, 2013**, and should be addressed to:

Ezra Heitowit

Executive Secretary for the Fermilab Director Search Committee
Fermi Research Alliance, LLC

Suite 400

1111 19th Street, NW

Washington, DC 20036 USA

e-mail: heitowit@fnal.gov

The two members of FRA are the University of Chicago and Universities Research Association, Inc. FRA operates Fermilab under contract with the U.S. Department of Energy. For more information about FRA and Fermilab visit <http://fra-hq.org> and <http://www.fnal.gov>, respectively.

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The Professorship of Statistics in Biomedicine

The Board of Electors to the Professorship of Statistics in Biomedicine invite applications for this Professorship from persons whose work falls within the general field of the Professorship to take up appointment on 1 October 2013 or as soon as possible thereafter.

Informal enquiries may be made to:

- **Professor Simon Tavaré, Professor of Cancer Research (Bioinformatics), Cancer Research UK Cambridge Institute,** simon.tavare@cruk.cam.ac.uk
- **Professor Sylvia Richardson, Director of MRC Biostatistics,** sylvia.richardson@mrc-bsu.cam.ac.uk, or
- **Professor John Todd, Acting Head of the Department of Medical Genetics,** john.todd@cimr.cam.ac.uk

Further information is available at: www.admin.cam.ac.uk/offices/academic/secretary/professorships/ or contact the Academic Secretary, University Offices, The Old Schools, Cambridge, CB2 1TT, (email: ibise@admin.cam.ac.uk), to whom a letter of application should be sent, together with details of current and future research plans, a curriculum vitae, a publications list and form CHRIS/6 (parts 1 and 3 only) with details of two referees, so as to reach him no later than **16 January 2013**.

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Tufts
UNIVERSITY

School of Medicine

Faculty Positions in Neuroscience

The Department of Neuroscience (www.neurosci.tufts.edu) at Tufts University School of Medicine is expanding by adding tenure-track faculty positions. Positions are available at **Assistant, Associate and Full Professor levels**. The department will build on its core strengths and focus on the study of synapses, disorders of the nervous system and neuron-glial interactions. We are particularly interested in building on our research that is relevant to epilepsy, depression, autism, neurodegenerative disorders and the neurobiology of obesity. We are seeking candidates who use innovative approaches to investigate problems that cross levels of investigation from molecular and cellular to systems and/or behavioral neuroscience. Candidates using molecular, genetic, electrophysiological and/or imaging methodologies to study neurons, synapses and networks are particularly encouraged to apply. We offer generous start-up packages, newly renovated laboratory space and a highly collaborative environment offering opportunities for both basic and translational research.

Applicants should hold a Ph.D. and/or M.D. degree and have several years of productive postdoctoral experience. Successful candidates will be expected to develop thriving, well-funded research programs and to contribute to graduate and medical education. Review of applications will begin **February 1st, 2013** and will continue until the position is filled. Please submit electronic applications including a CV, a statement of research interests and the names and email addresses of at least three references to: neurosci-facultyrecruitment@tufts.edu.

Tufts University is an Affirmative Action/Equal Opportunity Employer. We are committed to increasing the diversity of our faculty. Members of underrepresented groups are strongly encouraged to apply.

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ÉCOLE POLYTECHNIQUE
FÉDÉRALE DE LAUSANNE

Professorship in Energy Research and Director of the Energy Center at Ecole polytechnique fédérale de Lausanne (EPFL)

The Swiss Federal Institute of Technology, Lausanne (EPFL, <http://www.epfl.ch>) is a world-class European Research University and a growing, dynamic, public institution of higher education with a focus on engineering, computer science & communications, basic and life sciences.

EPFL is launching an international search and invites applications and nominations for the position of Director of its Energy Center to take office no later than 2014. The center director will also be appointed in one of the academic departments and is expected to establish a strong research program in her/his own area in addition to managing the Energy Center. EPFL provides strong institutional funding to support the research of its professors.

The Energy Center formulates a strategy and coordinates energy research activities on campus, it maintains a strong link to industry and its director is EPFL's main spokesperson in the energy domain. The Center provides the interface to the Swiss and European governmental and funding agencies. A broad spectrum of research is currently ongoing at EPFL in electrical power generation (e.g., hydroelectric, photovoltaic, fuel cells, solar fuels, wind, biochemical, plasma physics), energy storage (e.g., hydroelectric, batteries), and power networks and systems.

Major new initiatives with new buildings and positions in photovoltaics (at Neuchâtel) and renewable energies (in Valais) are planned. The Swiss

government has recently announced a major program to support energy research. EPFL is expected to be a key participant in this effort. The new Director of the Energy Center is expected to provide the intellectual and managerial guidance so that EPFL can help address the energy needs of Switzerland and the World.

The Director of the Energy Center will report directly to the VP for Innovation and Technology Transfer. The ideal candidate has an outstanding academic record, proven leadership, fundraising and negotiating skills, as well as knowledge transfer and management abilities.

The Search Committee invites applications (vision statement, complete CV and the names of at least 6 professional references) to be submitted at: <http://director-energyctr.epfl.ch>

Screening of dossiers will start **February 15th, 2013** and will continue until the position is filled.

Letters of nomination, expressions of interest or inquiries may be addressed confidentially to:

Professor Philippe Gillet
EPFL Provost & Chairman of the Search Committee
director.energyctr@epfl.ch

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- Interested in establishing a high-profile research programme? Through our research programme PEARL (financial contribution up to EUR 5 million) we give you the opportunity to transfer your research programme to a research institution in Luxembourg.



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More information about ATTRACT and PEARL as well as the other funding opportunities offered by the National Research Fund Luxembourg can be found on the FNR's website.

Go and see what's behind on www.fnr.lu/pearl and www.fnr.lu/attract

For an overview on research in Luxembourg, have a look at www.innovation.public.lu

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Inserm is the only French public research institute to focus entirely on human health. Its researchers are committed to studying all diseases, whether common or rare. Through its diversity of approaches, Inserm provides a unique environment for researchers. 13 500 researchers, engineers and technicians work in the 300 Inserm laboratories housed in hospitals, universities and research campuses, all over France.

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Research Directors: February 28th, 2013

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DIVISION HEAD for Hypertension and Vascular Research Division Henry Ford Health System (HFHS) – Detroit, Michigan

The Department of Internal Medicine at Henry Ford Hospital <http://www.henryford.com> is seeking applications for a Division Head of the Hypertension and Vascular Research Division (HVRD) http://www.henryford.com/body_program.cfm?id=52061. The HVRD has 7 staff investigators and over 30 support staff who conduct research studies on the role of vasoactive systems (autocrine, paracrine, and endocrine) in the regulation of cardiovascular and renal function, pathogenesis of hypertension, and mediation of end organ damage in hypertension. The Division has been supported by two NIH Program Project Grants, multiple R01s, foundation grants, and institutional funds. Total research funding in 2011 exceeded \$5 million from external sources. The Henry Ford Health System has a record of innovation and excellence in basic, clinical, epidemiological, and health services research, with external funding exceeding \$53 million in 2011, ranking first in NIH funding among non-university independent hospitals in Michigan and 19th in the country.

Position Description: The successful candidate should be a well-established scientist or physician scientist having expertise in hypertension and/or cardiovascular or renal pathophysiology, or expertise in a complementary field. The candidate should have a strong track record of NIH grant support. The Division Head will maintain his/her own NIH grants and research program and will be responsible for the Division's overall strategic direction and goals, as well as overseeing educational and mentorship activities related to the Division. The Division Head directly reports to the Chair of the Department of Internal Medicine. Appointment at Wayne State University School of Medicine is available commensurate with the applicant's background. Sufficient space and funds will be provided to ensure success of the program.

Basic Qualification: The candidate must have a Ph.D., M.D., M.D./Ph.D. or equivalent degree in a field relevant to the position. The candidate should possess recognized research management and leadership abilities, with the desire to foster relations between the laboratory and clinic.

Submission Instructions: Applicants should submit a cover letter, curriculum vitae, including list of publications, a description of their past and present research activities, and contact information for three references to: Sandra A. Rempel, Ph.D., Chair of the Search Committee, c/o Jennifer Feddersen, Henry Ford Hospital, Detroit, MI. Application materials should be sent to JFEDDER1@hfhs.org by March 1, 2013.



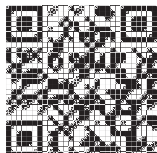
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In 2013, CNRS is recruiting 307 permanent researchers

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Online registration at www.cnrs.fr
from **December 3, 2012**
Registration deadline **January 7, 2013**



Assistant Professor Department of Biomedical Sciences

Cornell is a community of scholars, known for intellectual rigor and engaged in deep and broad research, teaching tomorrow's thought leaders to think independently, care for others, and create and disseminate knowledge with a public purpose.

The Department of Biomedical Sciences in the College of Veterinary Medicine at Cornell University invites applications for a tenure-track Assistant Professor Position in the area of Molecular Physiology. Most areas of physiology, with emphasis on one or more of the following areas: metabolism, metabolic disorders (e.g., diabetes mellitus, obesity, thyroid dysfunction), metabolomics, cardiometabolic disorders, endocrinology, and endocrine disorders. Candidates must utilize multi-scale integrated approaches that encompass genetic, molecular, cellular and whole animal systems to understand the complexity of cell and tissue function/dysfunction. The successful applicant should have a Ph.D., D.V.M., M.D., or equivalent degree, and will be expected to contribute to undergraduate Animal Physiology teaching.

The new faculty member will join the collaborative Life Sciences community on the main campus of Cornell University, in Ithaca, New York, with additional potential for interactions with Weill Cornell Medical College faculty in New York City. The position offers opportunities to participate in several campus-wide faculty-driven research initiatives, including those in Vertebrate Genomics and Stem Cell Science, to integrate with other units across both campuses, and to utilize the numerous state-of-the-art core facilities on campus. Please visit our Departmental website <http://www.vet.cornell.edu/biosci/> for additional information.

Nestled in the picturesque Finger Lakes region of upstate New York, Ithaca boasts a superb array of attractions and amenities in an environment that is supportive and nurturing for individuals and families alike. Cornell University embraces diversity and seeks candidates who will create a climate that attracts trainees of all races, ethnicities, and genders. Women and underrepresented minorities are strongly encouraged to apply. Cornell University also seeks to meet the needs of dual career couples, and is a member of the Upstate New York Higher Education Recruitment Consortium to assist with dual career searches. For more information, please contact Dr. Paula Cohen, Faculty Search Committee Chair, at molphysiology@cornell.edu. Prospective candidates should submit their application materials (cover letter, CV, Research and Teaching statements [3 pages each], up to 3 pertinent publications) via <https://academicjobsonline.org/ajo/jobs/1733> together with 3 letters of reference. The search committee will begin considering complete applications on January 18th and will continue until the position is filled.



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EBBE NIELSEN PRIZE Biodiversity Science/ Informatics

The Global Biodiversity Information Facility (GBIF) and its Science Committee invite nominations for 2013 for the prestigious Ebbe Nielsen Prize (€30,000) in *integrating biodiversity science and informatics*.

**Deadline for nominations:
15 February 2013**

For more information about the prize and nomination procedures, visit:

www.gbif.org

TENURE TRACK FACULTY POSITION IN CANCER RESEARCH

Case Western Reserve University School of Medicine Case Comprehensive Cancer Center

The Case Comprehensive Cancer Center (<http://cancer.cwru.edu/>), a National Cancer Institute-designated Comprehensive Cancer Center at CWRU, with affiliates at University Hospitals Case Medical Center and Cleveland Clinic, invites applications for tenure track faculty positions at the level of Assistant and Associate Professor in cancer biology. Candidates should have a doctorate and post-doctoral research experience. Candidates at the Assistant Professor level should provide a record of scholarly activity and external funding and have the potential to advance in cancer research. Candidates at the Associate Professor level should have a nationally-funded program and an outstanding record of cancer research achievements. Target areas of interest should be aligned with one of the Cancer Center's scientific programs, including regulation of cell proliferation and apoptosis, signal transduction, cell cycle regulation, DNA damage and repair, chromatin and epigenetics, cancer genetics, cancer stem cells, breast cancer, ovarian cancer, or colon cancer. Individuals with expertise in high through-put genomic methods are particularly encouraged to apply. Priorities include innovative discovery research coupled with an interest in translational clinical disease-oriented cancer research.

The successful candidate will have a primary appointment in the Cancer Center or a basic science department at the medical school such as Biochemistry (<http://www.case.edu/med/biochemistry/>), Molecular Biology & Microbiology (<http://www.case.edu/med/microbio/>), or Pharmacology (<http://pharmacology.case.edu/>).

Please send curriculum vitae, a list of three or more references, and a cover letter outlining your research interests electronically to: cancersearch@case.edu. Please include "Cancer Research Faculty Search" in the subject line.

In employment, as in education, Case Western Reserve University is committed to Equal Opportunity and Diversity. Women, veterans, members of underrepresented minority groups, and individuals with disabilities are encouraged to apply.

Case Western Reserve University provides reasonable accommodations to applicants with disabilities. Applicants requiring a reasonable accommodation for any part of the application and hiring process should contact the Office of Inclusion, Diversity and Equal Opportunity at 216-368-8877 to request a reasonable accommodation. Determinations as to granting reasonable accommodations for any applicant will be made on a case-by-case basis.



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